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Genetic and epigenetic modulation of p16[superscript INK4A] activity in human mammary epithelial cells provides insights into early events in breast cancer

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Genetic and Epigenetic Modulation of $p16^{INK4A}$ Activity in Human Mammary Epithelial Cells Provides Insights into Early Events in Breast Cancer

by

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DISSERTATION

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This dissertation is dedicated to
Alison, Andy, Mom, and Dad,
without whose support it
would not
exist

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At this juncture, I find myself (not surprisingly) with mixed emotions: the exhilaration of *being* "done," the exhaustion of *getting* "done," the profound satisfaction of "completing" a body of work, hope for my personal future and the future of biomedical research, gratitude for the guidance and support of all who have surrounded me the last half-decade, hesitance to part ways with my lab home-away-from-home, contentment that I have made good on a promise to myself.

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The text of Chapter Four of this dissertation is a reprint of the material as it appeared in *Cancer Research.*

The appendix of this dissertation is a reprint of the material as it appeared in *Nature.*

Thea D. Tlsty, the last co-author listed on each publication, directed and supervised the research that forms the basis for this dissertation.

Genetic and Epigenetic Modulation of $p16^{INK4A}$ Activity in Human Mammary Epithelial Cells Provides Insights into Early Events in Breast Cancer

Charles R. Holst

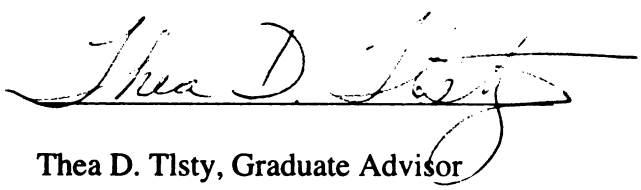
Abstract

Breast cancer is a major health concern of women worldwide. Breast cancer is typically derived from the epithelial cell component of the tissue, and is characterized by widespread chromosomal instability, as well as specific molecular lesions in known tumor suppressor genetic pathways. Human mammary epithelial cells (HMEC) isolated from healthy, disease-free women provide a physiologically relevant system in which to examine tumor suppression mechanisms.

Cultures of HMEC contain a subpopulation of variant cells with the capacity to propagate beyond an *in vitro* proliferation barrier. These variant HMEC (vHMEC), which contain hypermethylated and silenced $p16^{INK4a}$ (*p16*) promoters, eventually accumulate multiple chromosomal changes, many of which are similar to those detected in premalignant and malignant lesions of breast cancer. We show here that *p16* is necessary in HMEC to prevent proliferation beyond the *in vitro* proliferation barrier. Furthermore, consistent with its role as a regulator of cell cycle progression, *p16* activity is necessary for HMEC to arrest their cell cycle appropriately in response to microtubule disruption.

To determine the origin of vHMEC *in vitro*, we used Luria-Delbrück fluctuation analysis and found that vHMEC exist within the population prior to the proliferation

barrier, thereby raising the possibility that variant HMEC exist *in vivo* prior to cultivation. To test this hypothesis, we examined mammary tissue from normal women for evidence of *p16* promoter hypermethylation. Here we show that epithelial cells with methylation of *p16* promoter sequences occur in focal patches of histologically normal mammary tissue of a substantial fraction of healthy, cancer-free women. These observations show that molecular alterations historically associated with later stages in breast cancer progression occur much earlier than previously appreciated. They also validate the use of the HMEC culture system as a tool to stimulate translational research aimed at understanding the molecular pathology of breast cancer.



Thea D. Tlsty, Graduate Advisor

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CHAPTER 1

Introduction

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Breast Cancer is an Epithelial Cell Disease Characterized by Genomic Instability

Breast cancer is the most common malignancy among women in the United States, and is among the most common malignancies to afflict women in the world (www.cancer.org, 2003). Nearly all cancer arising in the mammary gland originates in the epithelial cell component of the tissue. This bias toward carcinomas is an epidemiological observation currently without satisfactory explanation.

Another nearly universal feature of breast carcinomas, as well as carcinomas in general, is the presence of highly aberrant genomic complements. Indeed, this underlying genetic instability has long been proposed to provide heterogeneity in an evolving neoplasm, from which sequential selection of variant subpopulations with increasingly aberrant phenotypes can evolve (Nowell, 1976). Karyotypic analysis of breast carcinomas has shown a tremendous quantity and diversity of chromosomal abnormalities (Teixeira et al., 2002). Large-scale searches for sites of loss-of-heterozygosity have further shown that chromosome instability is rampant in invasive breast cancer (Shen et al., 2000). Extrapolation from molecular analyses involving unbiased PCR-based approaches estimate that, in colorectal cancers, each tumor cell may contain >10,000 of the chromosomal defects detectable by these methods (Basik et al., 1997; Peinado et al., 1992; Stoler et al., 1999). Such revealing studies strongly suggest that tumor cells have evaded or inactivated whatever mechanisms exist in normal cells (*i.e.*, cell cycle checkpoints) to prevent such genomic anarchy.

Two Critical Tumor Suppressor Pathways: *p53* and *Rb*

The prevalence of breast cancer has prompted an intense search for underlying molecular defects that contribute to initiation and progression of the disease. Important insights have come from the study of families with high incidence of breast cancer cases (Fearon, 1997). Individuals with classic Li-Fraumeni syndrome, who possess germ-line mutations in the *p53* tumor suppressor gene, have a high frequency of pre-menopausal breast cancer, among other malignancies (Malkin et al., 1990; Srivastava et al., 1990). Extensive analysis of sporadic, non familial-associated, breast cancers has supported the role of *p53* in breast cancer. Nearly 30% of sporadic breast cancers possess *p53* mutations (Greenblatt et al., 1994).

Other genes in the *p53* pathway, including *ATM* and *Chk2*, are also mutated in the germlines of certain families at high risk for developing cancer. Heterozygote individuals carrying inherited mutations of the *ATM* (ataxia telangiectasia mutated) gene, as well as individuals with germ-line mutations in the *Chk2* gene (in Li-Fraumeni syndrome families without p53 mutations), are also at greatly increased risk for breast cancer and other cancers (Bell et al., 1999; Rotman and Shiloh, 1998). Study of inherited breast cancer susceptibility has also resulted in the discovery of the *BRCA1* and *BRCA2* genes (Venkitaraman, 1999), additional genes that also interact with members of the *p53* pathway.

Extensive research into the molecular genetic underpinnings of cancer has revealed another pathway critical for tumor suppression: the *Rb* genetic pathway. The *Rb* pathway functions to modulate entry into the DNA synthesis phase of the cell cycle by affecting the activity of the E2F transcription factor family (Wang et al., 1994a;

Weinberg, 1995). The components of the *Rb* pathway that have been implicated in carcinogenesis are *cyclin D1*, *Cdk4*, and $p16^{INK4a}$. Cdk4 is a cyclin-dependent kinase that complexes with the D-type cyclin cofactors to phophorylate the Rb protein, thereby inhibiting the ability of Rb to inhibit the activity of E2F. Farther upstream, $p16^{INK4a}$ inhibits the activity of the Cdk4 kinase. The *Rb* gene was the first tumor suppressor gene to be cloned (Friend et al., 1986). Individuals that inherit a single copy of an inactivating point mutation of *Rb* develop childhood retinoblastoma tumors with ~95% penetrance (Knudson, 1971). Furthermore, offering validation of Knudson's two-hit model of tumor suppressor inactivation, careful analysis of the retinoblastoma tumors in these patients revealed that the second allele of the *Rb* gene had also been inactivated, by novel intragenic mutation, gene deletion, chromosomal loss, or somatic recombination (Cavenee et al., 1983).

The *p16* gene resides at human chromosomal position 9p21, a region frequently deleted in tumors (Kamb et al., 1994; Nobori et al., 1994). Subsequent linkage analysis studies identified familial melanoma kindreds with germline mutations in the *p16* gene (Gruis et al., 1995), confirming its status as a tumor suppressor gene. p16 was originally identified as a protein that physically associated with the cyclin D-dependent kinase Cdk4 (Serrano et al., 1993; Xiong et al., 1993). Indeed, alterations in all three of these physically and functionally interacting gene products (p16, cyclin D, and Cdk4) have been associated with familial and/or sporadic tumor formation.

Inactivation of *p16* occurs in many different tumor types, with mechanism(s) and frequency of inactivation varying among different tumor types. The mechanisms of *p16* inactivation are both genetic (deletion or intragenic point mutation) and epigenetic

(promoter hypermethylation-associated gene silencing) in nature (Sherr and McCormick, 2002). For instance, over 90% of pancreatic carcinomas contain *p16* inactivation (48% by homozygous deletion, 34% by hemizygous deletion and point mutation, and 16% by hemizygous deletion and methylation-associated silencing) (Schutte et al., 1997).

Compared to pancreatic carcinomas, *p16* inactivation is less frequent in invasive breast cancers, detected in ~30% of tumors (Sherr and McCormick, 2002). Approximately 25% of breast tumors contain *p16* promoter hypermethylation (Esteller et al., 2001; Merlo et al., 1995). However, an additional ~50% of breast carcinomas overexpress cyclin D1 (Bartkova et al., 1994; Gillett et al., 1994; McIntosh et al., 1995). ~20% of breast tumors overexpress cyclin D1 due to gene amplification (Dickson et al., 1995). The molecular mechanism of cyclin D1 overexpression in the remaining~30% of breast carcinomas remains elusive. Overexpression of cyclin D1 has been observed in ductal carcinoma *in situ* lesions, but not in hyperplastic lesions (Weinstat-Saslow et al., 1995), suggesting an early role for cyclin D1 dysregulation in breast cancer progression.

As is evident in the breast cancer case, loss of function of the *Rb* pathway may occur by a number of different mechanisms. For reasons currently unknown, the apparent preferred mechanism of *Rb* pathway inactivation differs among tumors originating in different tissues. In retinoblastomas and small-cell lung carcinomas, *Rb* is frequently mutationally inactivated. In high-risk human papilloma virus-associated cervical carcinomas, the E7 gene product of the virus inactivates Rb. Additional mechanisms of Rb pathway inactivation include (a) *cyclin D1* amplification and/or overexpression common to esophageal and breast carcinomas, (b) *Cdk4* amplification in glioblastoma multiforme, (c) activating *Cdk4* point mutations in melanomas, (d) inactivating $p16^{INK4a}$

point mutations in sporadic and hereditary melanomas, and (e) hypermethylation-mediated silencing of $p16^{INK4a}$ in a variety of different tumors (Chin et al., 1998).

In support of the *pathway* being a single mutational target, lesions in *Rb*, *Cdk4*, and $p16^{INK4a}$ are usually mutually exclusive in a single tumor (Ruas and Peters, 1998; Sherr and McCormick, 2002). For example, loss of p16 expression is observed in 30-50% of non small cell lung cancer tumors, whereas Rb expression was lost in an additional 15-30%, with very few tumors exhibiting both defect (Ruas and Peters, 1998). Another example is provided by the analysis of glioblastomas, wherein ~15% of tumors show amplification of *Cdk4*, with this molecular abnormality being detected, in large part, in different tumors than those that contain *p16* or *Rb* alterations (Ruas and Peters, 1998). Exceptions to this mutual exclusivity have certainly been reported, but the general trend remains true.

Experimental Evidence that the *p53* and *Rb* Pathways are Indeed Tumor Suppressive in the Mammary Gland: Mouse Models of Cancer

Observational studies of the molecular defects in human tumors offer plentiful suggestive evidence for the involvement of candidate molecules in the neoplastic process. The importance of such suggestive evidence can be bolstered substantially by parallel studies in experimental systems. Due largely to the extensive genetic tools available, the experimental system of choice to many cancer researchers has been the laboratory mouse. The experimental evidence that the *p53* and *Rb* pathways are generally tumor suppressive in the mouse is extensive (Donehower et al., 1992; Harvey et al., 1993; Jacks et al., 1992; Jacks et al., 1994; Williams et al., 1994), and will not be summarized here. However, the evidence that these two tumor suppression pathways are indeed involved in preventing mammary gland neoplasia will be considered here.

Mice lacking the *p53* tumor suppressor are viable and survive to adulthood, whereupon they begin to succumb to spontaneously arising tumors, which are predominantly lymphomas and soft-tissue sarcomas (Donehower et al., 1992; Donehower, 1996; Jacks et al., 1994). In contrast to the human epidemiological observation that women with germline mutations in *p53* develop mammary carcinomas with high frequency (Malkin et al., 1990), mammary carcinomas in the $p53^{-/-}$ and $p53^{+/-}$ mice (in the original C57Bl/6×129/Sv genetic background) were very rarely observed (Donehower et al., 1992; Donehower, 1996; Jacks et al., 1994), an observation which may have caused some consternation among those who hoped the mouse phenotype would phenocopy the human phenotype.

The development of two refined model systems has since shown the importance of wild-type *p53* in suppressing mammary carcinogenesis in the mouse, and improves the attractiveness of using these model systems to study the role of *p53* in mammary carcinogenesis. The first system to reveal the role of *p53* in suppressing mammary tumor formation was a study in which the $p53^{null}$ allele was transferred into the BALB/c genetic background (Kuperwasser et al., 2000), a mouse strain chosen because female BALB/c had previously been shown to be sensitive to radiation-induced mammary carcinogenesis (Ponnaiya et al., 1997; Ullrich et al., 1996). Amazingly, 42% of the tumors observed in the $p53^{+/-}$ BALB/c mice were mammary carcinomas. Indeed, the tumor spectrum of the female BALB/c *p53* heterozygote mice mimics the tumor spectrum of women with Li-Fraumeni syndrome (Akashi and Koeffler, 1998; Kleihues et al., 1997; Kuperwasser et al., 2000; Malkin et al., 1990).

The altered tumor spectrum of the $p53^{null}$ allele in different genetic backgrounds suggests the presence of modifying loci in the different inbred mouse strains. Interestingly, the BALB/c strain of laboratory mice reportedly contains a hypomorphic allele of *p16* (Herzog et al., 1999; Zhang et al., 1998), which raises the possibility that the altered tumor spectrum in the BALB/c background (particularly the prevalence of mammary carcinomas) is due simply to the hypomorphic *p16* status. In other words, perhaps the coexisting *p16* and *p53* defects in BALB/c $p53^{+/-}$ animals cooperate to allow mammary carcinogenesis. Alternatively, perhaps other modifying loci in the BALB/c genetic background, independent of *p16*, are affecting the frequency of mammary carcinoma formation. The latter possibility is supported by a recent study (Sharpless et al., 2002), wherein mice (in a mixed genetic background; ~87.5% FVB/n) deficient in

p53 and/or *p16* were examined for tumor incidence and tumor spectrum. In this study, *p53* and *p16* indeed cooperated to prevent tumor formation in the whole organism (*i.e.*, animals containing null alleles for both genes developed tumors earlier than animals with only one or the other genetic deficiency). However, mammary carcinomas were still not observed, though the small number of animals examined in this study prevents a definitive resolution of this question. These results do however suggest that the "mammary carcinoma" modifying locus in the BALB/c background is distinct from *p16*.

p53 has also been shown to act as a mammary tumor suppressor in a mouse model which mimics the progressive telomere shortening thought to occur in human tissues during the aging process. Mice possessing *p53* knock-out mutations in the context of cells with experimentally shortened telomeres (due to multiple generation breeding of animals with a genetic deficiency of the *Terc* RNA subunit of telomerase) exhibit increased mammary carcinoma incidence (Artandi et al., 2000). Telomerase is a ribonucleoprotein complex involved primarily in the maintenance of the telomeric DNA repeats. In the absence of telomerase, proliferating mammalian cells undergo progressive telomere shortening. The lack of telomerase activity and/or the presence of shortened telomeres in late-generation $Terc^{-/-}$ mice dramatically alters the incidence and tumor spectrum of $p53^{+/-}$ animals, including a dramatic increase in the frequency of carcinomas observed (Artandi et al., 2000). The authors of these studies hypothesized that the chromosomal instability permitted by telomere shortening and/or lack of telomerase fuels epithelial tumor formation in the context of *p53* heterozygosity. Therefore, *p53* is indeed a breast cancer tumor suppressor, but such a role is only revealed under certain informative conditions in mouse models.

The experimental evidence that the *Rb* pathway is implicated in suppressing mammary gland tumor formation in mice is several-fold. Early suggestions came from the report of the *cyclin D1* knockout mouse phenotype, which was surprising and revealing in just how tissue-specific the defects were (Fantl et al., 1995; Sicinski et al., 1995). Among other phenotypes, *Cyclin D1*$^{-/-}$ mice lack a complete lobuloalveolar development of the mammary gland during late pregnancy, indicating a role for *cyclin D1* in the proliferative response of normal mammary epithelial cells under physiological conditions (Fantl et al., 1995; Sicinski et al., 1995). In an attempt to mimic the frequent overexpression of cyclin D1 in human mammary carcinomas, it was subsequently shown that transgenic overexpression of cyclin D1 in a mammary gland-specific pattern was sufficient to cause mammary carcinomas (Wang et al., 1994b). The definitive evidence that *cyclin D1* is indeed necessary for tumor development was reported recently (Yu et al., 2001). Sicinsky and colleagues showed that loss of *cyclin D1* (due to genetic ablation) specifically prevented the tumor formation induced by transgenic mammary gland-specific expression of either the c-*neu* or v-Ha-*Ras* oncogenes. Therefore, under those experimental conditions, endogenous *cyclin D1* is needed for tumor formation, validating its role as a tumor-promoting gene. In an intriguing observation, it was shown that *cyclin D1* status had no effect on the incidence or progression of either *Wnt-1* or c-*myc* oncogene-driven breast carcinomas (Yu et al., 2001). *In toto*, experiments examining the role of *cyclin D1* in mouse models of mammary gland carcinogenesis have validated its role under certain tumor conditions, and underscore the importance of the clinical observation of cyclin D1 overexpression in the majority of human breast cancers.

To date, neither the *pRb* knockout or *p16* knockout mice have been reported to exhibit increased incidence of mammary cancers (Jacks et al., 1992; Krimpenfort et al., 2001; Sharpless et al., 2001). This indicates that either these genes are not involved in mammary cancer in the mouse, or, as was true in the case of *p53*, the experimental conditions under which to reveal the role of *p16* and *pRb* as mammary gland tumor suppressors have yet to be uncovered. For instance, as was indicated in the case of *p53* (Artandi et al., 2000), perhaps telomerase inactivation is needed to allow mammary carcinoma development in the mouse. The phenotypic description of the late generation $Terc^{-/-}$; $p16^{-/-}$ double mutant is thus anxiously awaited.

Plentiful experimental evidence implicates the *p53* and *pRb* pathways in suppressing the formation of mammary gland carcinomas in the laboratory mouse. A greater understanding of how these genetic pathways function in normal human cells to modulate phenotypes hypothesized to be tumor suppressive is therefore fundamental to a full explication of why these pathways are so commonly targeted in tumors of different types.

Cell Cycle Checkpoint Control as a Possible Tumor Suppression Mechanism

The proper timing and coordination of events in the cell duplication cycle are critical for the maintenance of the integrity of the genome. As originally shown by Weinert and Hartwell, certain evolutionarily conserved genetic pathways exist in cells to ensure the coordination of various cell division cycle activities (Hartwell and Weinert, 1989). These pathways 'check' to make sure events are coordinated, and hence were termed 'checkpoint' pathways. The genetic underpinnings of cell cycle checkpoints in response to DNA damage have been revealed by extensive study in various model systems, including the fungi *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.* Intriguingly, many of the genes identified in these yeast systems are conserved evolutionarily, with structurally and functionally conserved homologs (including the aforementioned human tumor suppressors *ATM*, *Chk2*) present in many different multicellular eukaryotes. Highly conserved checkpoint genes such as *RAD9* function to prevent cell cycle progression in response to DNA damage (Weinert and Hartwell, 1988). *rad9* mutant cells are viable and do not possess overt phenotypic defects when proliferating in optimal conditions, a hallmark of checkpoint mutants (Hartwell and Weinert, 1989). However, when challenged by DNA damage, mutant cells continue cell cycle progression in the presence of persistent DNA damage, and cells eventually die (Weinert and Hartwell, 1988). More recent analysis of a different class of checkpoint genes (typified by *MEC3* and *RFC5*) has proposed that checkpoints also function to delay cell cycle progression in response to endogenously generated damage such as arises during DNA synthesis (Myung et al., 2001). A prominent phenotype of checkpoint deficient cells is the propensity toward the acquisition of genomic instability (Myung et

al., 2001; Paulovich et al., 1997). Stated another way, checkpoint pathways act to suppress genomic instability.

In addition to the highly conserved checkpoint genes, other components of mammalian checkpoint pathways, including *p53* and *Rb*, lack clear homologs in unicellular model systems, and hence must be studied in other systems, such as *Drosophila*, the laboratory mouse, and human cell culture. I will now discuss the hypothesized role of the *Rb* and *p53* pathways in cell cycle checkpoint pathways of mammalian cells.

Rb functions primarily to regulate the initiation of DNA synthesis. Such cell cycle progression is governed by the coordinated activity of highly conserved cyclin-dependent kinase (CDK) complexes. Entry into S (DNA synthesis) phase of the cell division cycle in higher eukaryotes correlates with the functional inactivation of Rb by CDK-mediated phosphorylation (Chin et al., 1998; Sherr, 1996). Such progression is regulated (a) positively by the D-type and E-type cyclin cofactors and (b) negatively by a variety of CDK inhibitors, including members of the Cip/Kip and INK4 families. Although the details of the interactions are considerably more complex (Dyson, 1998), the prevailing model is that when CDK activity is low, Rb is in a largely hypophosphorylated state, allowing the inhibitory association of Rb with certain transcriptionally 'activating' members of the E2F transcription factor family (Chin et al., 1998; Trimarchi and Lees, 2002). When Rb is hyperphosphorylated through the coordinated action of CDK/cyclin complexes, Rb no longer associates with these E2F's, which are then free to transactivate target genes necessary for DNA synthesis. In addition to being a negative regulator of E2F-dependent transcription transactivation, Rb (and/or the related p107 and p130 family

members) can form complexes with other 'repressive' E2F's, acting together to repress gene expression largely via the recruitment of histone deacetylases (HDAC) (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Stevaux and Dyson, 2002; Trimarchi and Lees, 2002).

p16 was the founding member of the INK4 family of cyclin-dependent kinase inhibitors (CKI). Among the various cyclin-dependent kinase activities, the INK4 inhibitors act preferentially to inhibit the G1-associated D-type cyclin-associated kinases, Cdk4 and Cdk6. Cdk4/6 are enzymatically inactive in the absence of their cyclin D cofactor partners. *In vitro* binding assays and follow-up *in vivo* studies revealed that p16 protein binds to Cdk4/6 directly, thereby preventing cyclin association, and by extension, kinase activity (Serrano et al., 1993; Xiong et al., 1993).

Cell cycle progression is also negatively regulated by the Cip/Kip family of CKI. The Cip/Kip family (which includes $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) was originally described to inhibit cyclin D-, E-, and A-dependent kinases *in vitro*. This view was challenged by the observation that, although they indeed potently inhibit Cdk2, which pairs with cyclins A and E, at low levels the Cip/Kip proteins can act as positive regulators of the cyclin D-associated kinases (Cheng et al., 1999).

p53 also functions to regulate cell cycle progression, in part due to its ability to transcriptionally transactivate the aforementioned $p21^{Cip1}$ CKI gene. In a cell type-specific manner, p53 is capable of initiating apoptotic cell death in response to a variety of cellular damage situations (Sherr and McCormick, 2002), which may also contribute to its function as a tumor suppressor. Here I will focus on p53's role in cell cycle checkpoint control. Although p53 has been attributed with a plethora of different biological

activities, prominent among these is its well-characterized role as a transcription factor (Levine, 1997; Prives and Hall, 1999). Indeed, many tumor-associated mutations in *p53* obliterate its activity as a transcriptional activator (Hollstein et al., 1991). Consistent with its role in a checkpoint capacity, the transcriptional activity of p53 is stimulated by various forms of DNA damage, and this activity is critical to prevent cell cycle progression in response to such damage. To address the role, if any, of the transcriptional activation activity of p53 in tumor suppression, a 'knock-in' mouse was recently generated in which the wild-type allele of *p53* was replaced with a transcriptionally inactive mutant allele (Jimenez et al., 2000). Reminiscent of the $p53^{-/-}$ phenotype described above (Donehower et al., 1992), this mutant mouse was also tumor prone (Jimenez et al., 2000), which suggests that the ability of p53 to transactivate transcription is necessary for its tumor suppressive activity. The Cdk inhibitor, *p21*, is a prominent transcriptional target of *p53*, and is partially responsible for the DNA damage-induced *p53*-dependent cell cycle arrest (Brugarolas et al., 1995; Deng et al., 1995). However, *p21* deficent mice are not tumor-prone; hence, additional transcriptional targets or biological activities of *p53* must be sufficient for its role as a tumor suppressor.

Functional evidence that *p53* and *Rb* act in cell cycle checkpoint control pathways is extensive. For instance, although early passage $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) do not display obvious proliferation defects, they are indeed highly susceptible to genotoxic agents and fail to arrest cell cycle progression after DNA damage, induced telomeric damage, and other experimental treatments (Karlseder et al., 1999; Kuerbitz et al., 1992). The checkpoint function of *p53* has been strongly supported in human cells as well (Kastan et al., 1991; Kastan et al., 1992; Kuerbitz et al., 1992). Furthermore, the *p53*

status of MEFs and human fibroblasts critically affects their ability to prevent genomic instability, as assayed by the CAD gene amplification system (Livingstone et al., 1992; Yin et al., 1992). Although $Rb^{-/-}$ MEFs possess no overt proliferation defects, these mutant cells do possess dramatic cell cycle checkpoint defects in response to various damaging agents (Harrington et al., 1998; Sage et al., 2000). For instance, $Rb^{-/-}$ MEFs fail to prevent cell cycle progression in response to DNA damage induced by gamma radiation (Harrington et al., 1998). Like *p53*, *Rb* thus has characteristics of a checkpoint gene.

A Specialized Case of the Regulation of Cell Cycle Progression: Control of Replicative Potential

In addition to regulating cell cycle progression in response to acute damage, the *p53* and *Rb* pathways have also been implicated extensively in the prevention of immortalization of normal human cells. The concept of human somatic cell "mortality" was first suggested by the pioneering studies of Leonard Hayflick, who first showed that fibroblast cells divide a limited number of times before ceasing proliferation at a population growth plateau variously called the Hayflick limit, mortality stage 1 (M1), or replicative senescence (Hayflick, 1965; Wright et al., 1989). Subsequent studies have shown that the spontaneous immortalization frequency of newborn skin fibroblasts is extremely low (conservatively estimated at $<10^{-9}$); indeed, spontaneous fibroblast immortalization has never been observed (McCormick and Maher, 1988). The replicative senescence population growth plateau is a *bona fide* cell cycle arrest, in that senescent fibroblasts exhibit low proliferation and death indices (*e.g.*, (Romanov et al., 2001); Figure 1-1, *A*). Furthermore, senescent fibroblast cultures arrest in the presence of a normal karyotype (Bischoff et al., 1990).

Changes in telomeric function are thought to trigger the replicative senescence arrest. Upon continued proliferation in culture, human cells exhibit a constant shortening of the telomeric repeats at the chromosome termini. Indeed, in many fibroblast strains, replicative senescence can apparently be averted by the ectopic expression of telomerase (Bodner et al., 1998; Vaziri and Benchimol, 1998), the primary ribonucleoprotein complex responsible for maintenance of telomeres *in vivo*. These results also argue that some condition brought about by proliferation in the absence of telomere maintenance

(*i.e.*, telomere shortening or telomere dysfunction) may trigger the replicative senescence arrest.

Two lines of evidence suggest that replicative senescence is dependent on the *p53* and *Rb* checkpoint pathways. First, various different oncoproteins from immortalizing viruses, including E6 and E7 from high risk human papilloma viruses, the large T antigen from SV40, and the E1A and E1B adenovirus gene products, all target p53 and Rb, among other proteins, for inactivation. Expression of such viral oncoproteins in normal skin fibroblasts extends the lifespan of the cell cultures. For example, expression of HPV 16 E6, which targets p53 for inactivation, delays fibroblast senescence ~10-15 population doublings (PD) (White et al., 1994). E7 alone also extends *in vitro* lifespan by ~5-10 PD (White et al., 1994). Co-expression of E6 and E7 in fibroblasts extends lifespan >20 PD, at which time the population temporarily ceases expansion (White et al., 1994). However, in sharp contrast to the static cell cycle arrest of the senescence plateau, fibroblasts expressing E6/E7 encounter a dynamic state termed crisis or mortality stage 2 (M2), during which abundant proliferation and death occur (Wright et al., 1989). Another prominent characteristic of cells in crisis is the presence of abundant genomic abnormalities (Counter et al., 1992; Ray and Kraemer, 1993). With a frequency of $\sim 10^{-7}$, immortal clones arise from the crisis plateau. Importantly, the E6 and E7 gene products are both capable of disrupting cell cycle checkpoint control in pre-crisis fibroblasts, as well as increasing the frequency of genomic changes such as gene amplification (as measured by amplification of the endogenous CAD gene locus in (White et al., 1994)).

Second, because the viral oncoproteins utilized in the early studies are multifunctional, several attempts have been made to disrupt *p53* and *Rb* in a more

specific manner to address their role in the prevention of human fibroblast immortalization. Treatment of fibroblast cultures with antisense oligonucleotides to inhibit *p53* and/or *Rb* specifically support the requirement of their gene products in enforcing senescence (Hara et al., 1991). In an effect similar to the expression of E7, inhibition of *Rb* by antisense methods resulted in an extension of lifespan by ~10 PD (Hara et al., 1991). Although antisense inhibition of *p53* did not delay the growth plateau, antisense inhibition of both *p53* and *Rb* was more effective than *Rb* antisense alone, arguing that *p53* does play a role in human fibroblast senescence (Hara et al., 1991).

Further evidence implicating the *p53* and *Rb* pathways in replicative senescence of human fibroblasts comes from experiments disrupting other components of these pathways. Human fibroblasts wherein the *p21* gene (a primary transcriptional target of the p53 transcription factor) has been inactivated by targeted homologous recombination exhibit an extended lifespan, proliferating beyond when control cultures senesced (Brown et al., 1997). A second study showed that senescence is delayed in fibroblasts with *p16* inhibited by antisense techniques (Duan et al., 2001).

Although inhibition of *p53* and *Rb* delays fibroblast senescence, the cell populations do eventually cease to increase in number (Hara et al., 1991). This indicates that inactivation of *p53* or *Rb* alone or together is not sufficient to immortalize human cells. Indeed, due to the low frequency (10^{-7}) of immortalization of viral oncoprotein-transduced fibroblasts, additional genetic or epigenetic events appear to be required for full immortalization. If additional events were *not* required, then the frequency of immortalization when *p53* and *Rb* were inactivated would approach 100%. A central candidate among these hypothesized additional event(s) necessary for immortalization of

human fibroblasts is the reactivation of telomerase (or some other means by which telomeres are stabilized); this hypothesis is supported by experimental observation (Montalto et al., 1999), in that immortalized clones arising from viral oncoprotein-induced crisis all exhibit telomere maintenance.

In conclusion, decades of research aimed at elucidating the molecular genetics underlying cancer initiation and progression have revealed several common, if not universal, molecular features of tumor cells. These include, but are not limited to, molecular alterations in the *Rb* and *p53* genetic pathways. These molecular changes affect not only the regulation of cell cycle progression and, by extension, the regulation of genomic stability, but also play critical roles in limiting the replicative lifespan of normal cells.

A Human Mammary Epithelial Cell Culture System in which to Study Tumor Suppressive Phenotypes

Because of the bias toward epithelial cell-derived tumors, epithelial cell culture systems may provide better model systems for the study of carcinogenesis than fibroblast-based systems. Several complementary systems have been developed for the culture of human mammary epithelial cells *in vitro*. These culture systems differ in their medium formulations, digestion treatment of primary tissue, and presence or absence of feeder layers (Kao et al., 1995; Péchoux et al., 1999; Stingl et al., 2000). In our laboratory, we have utilized the cell culture approach developed by Dr. Martha Stampfer (Hammond et al., 1984; Stampfer, 1982; Stampfer et al., 1980). The Stampfer approach to HMEC propagation in culture is the most commonly used HMEC system, and the commercial availability of cells and culture medium expedite such use.

When cultured in the standard serum-free medium, MCDB 170, HMEC exhibit a growth curve that differs substantially from the standard fibroblast curve. The growth curve consists of two periods of exponential proliferation, punctuated by two distinct population growth plateaus (Fig. 1-1, *B*). HMEC populations exhibit *in vitro* proliferation behavior different than the classical replicative senescence behavior of isogenic human mammary fibroblasts (HMF; Romanov et al., 2001; Tlsty et al., 2001). After 10-15 population doublings in culture, the overwhelming majority of HMEC encounters a proliferation barrier and activates a cell cycle arrest that is *phenotypically* similar to fibroblast senescence (normal karyotype, low proliferation and death indexes (Romanov et al., 2001)). This first population growth plateau has been called a variety of terms by different research groups, including self-selection, mortality stage 0 (M0), and

senescence (Foster and Galloway, 1996; Hammond et al., 1984; Huschtscha et al., 1998). For ease of use, and to avoid mechanistic implications, in this document, I will attempt to refer to this proliferative arrest of HMEC as the first plateau.

In stark contrast to the extraordinarily low frequency of spontaneous escape from senescent fibroblast populations ($<10^{-9}$), the frequency with which HMEC capable of proliferating beyond the first plateau arise is quite high (10^{-4}-10^{-6}) (Holst et al., 2003; Romanov et al., 2001). In this document, I will refer to the distinct subpopulation that proliferates beyond the first plateau as variant HMEC, or vHMEC. At the first plateau, vHMEC appear as colonial outgrowths of small cells among the background of large, vacuolated, non-proliferating cells (Hammond et al., 1984; Romanov et al., 2001). vHMEC are characterized by low p16 expression, due to promoter hypermethylation-mediated silencing of *p16* gene expression (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998; Wong et al., 1999). vHMEC have been characterized also by a host of additional changes in gene expression, including increases in p53 and p21 protein levels (Delmolino et al., 1993; Romanov et al., 2001).

Previous work has suggested that the emergence of proliferating mammary epithelial cells from the HMEC first plateau is dependent on the status of the *p16/Rb* pathway, and seemingly independent of *p53* status. First, HMEC populations at the first plateau are characterized by increased expression of p16 (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998; Romanov et al., 2001). Second, as previously mentioned, vHMEC that proliferate beyond the first plateau exhibit low expression levels of the p16 protein, predominantly by hypermethylation of CpG islands in the *p16* promoter (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998). Third,

expression of the viral oncoprotein, HPV16 E7, which functionally inactivates the Rb protein, but not HPV16 E6, which targets p53 for degradation, appears to prevent the first plateau (Foster and Galloway, 1996). Doubts have been raised, however, about the specificity of the effect(s) of E7 expression, as this viral protein has been shown to interact functionally with many cellular proteins apart from Rb, including p21 and p27 (Funk et al., 1997; Zerfass-Thome et al., 1996). It has yet to be shown that loss of p16 is necessary or sufficient for continued proliferation beyond the proliferative arrest.

Subsequent to the first plateau, vHMEC proliferate an additional 30 – 50 generations beyond the time that the bulk population activates the proliferative arrest. vHMEC undergo progressive telomere shortening and eventually reach a second population growth plateau we previously termed agonescence (Romanov et al., 2001; Tlsty et al., 2001), which is phenotypically different from human mammary fibroblast senescence and the HMEC first plateau. Agonescent vHMEC populations have both moderately high proliferation and death indexes, although they exhibit no net increase in cell number (Romanov et al., 2001). Furthermore, in an unprecedented observation in "normal," unmanipulated human cells, nearly 100% of vHMEC approaching agonescence exhibit chromosomal defects, including aneuploidy, telomeric associations, and various other classes of structural abnormalities (Romanov et al., 2001). Such chromosomal instability is reminiscent of the abundant and heterogeneous chromosomal changes observed in pre-malignant and malignant breast cancer lesions (Shen et al., 2000; Teixeira et al., 2002), as well as the chromosomal changes that accompany viral oncoprotein-induced crisis in fibroblasts (Counter et al., 1992; Ray and Kraemer, 1993).

Two sets of studies initially suggested a role for p53 in preventing mammary epithelial cell immortalization. First, expression of HPV16 E6 is sufficient to immortalize vHMEC populations, seemingly without a "crisis" event (Shay et al., 1993; Wazer et al., 1995). Second, mammary epithelial cells derived from a Li-Fraumeni syndrome patient (heterozygous for a missense mutation in the *p53* gene) exhibited a low, but reproducibly detectable, frequency of immortalized cell populations (Shay et al., 1995).

The role of p53 in suppressing mammary epithelial cell immortalization was cast into doubt, however, by the observation that E6 mutants defective in their ability to inactivate p53 were still capable of efficiently immortalizing vHMEC cultures (Kiyono et al., 1998). In a surprising observation, it was shown that E6, in an epithelial cell-specific manner, causes activation of telomerase; this activity of E6 appears to be essential for efficient vHMEC immortalization (Kiyono et al., 1998). Indeed, ectopic expression of the telomerase catalytic protein subunit (hTert) is sufficient to immortalize vHMEC efficiently (Kiyono et al., 1998).

Aims of this Dissertation

The human mammary epithelial cell culture system has provided us with a unique system in which to examine hypothesized tumor suppressive mechanisms in a physiologically and potentially pathologically relevant scenario. The purpose of my research project has been four-fold:

First, as described in Chapter 2, using the new experimental tool of RNA interference, we show that *p16* is indeed necessary for the first plateau. We also show that the *p16* and *p53* pathways are interconnected in HMEC, but not isogenic mammary fibroblasts, providing possible insight into cell type-specific regulation of these important tumor suppressor genes.

Second, as shown in Chapter 3, I sought to explore the integrity of cell cycle checkpoint control in mammary cells. Here I describe that vHMEC, which are deficient in *p16* expression, exhibit two specific cell cycle checkpoint defects: (i) in contrast to isogenic HMEC and HMF, vHMEC fail to maintain a gamma radiation-induced DNA damage cell cycle checkpoint arrest, and (ii) vHMEC also lack a microtubule integrity-sensitive 2N phase cell cycle arrest. In an interesting twist, the *p16* dependence of these two checkpoints differs, in a cell type- and checkpoint-specific manner. These results raise two important subtleties, namely that *p16* gene function can differ from cell type to cell type, and that not all emergent phenotypes of vHMEC are due simply to *p16* deficiency.

Third, in the first section of Chapter 4, I sought to examine the origin of the vHMEC population. To achieve this, I utilized a statistical analysis tool called Luria-Delbrück Fluctuation Analysis to ask the question, "Are vHMEC induced by the first

plateau; or do they pre-exist before the first plateau, and are selected for at the plateau?" In short, I showed that the vHMEC subpopulation (or a precursor thereof) exists prior to the proliferative arrest of the first plateau. This raised the possibility that vHMEC (cells characterized by hypermethylated *p16* promoters) exist in the primary reduction mammoplasty tissue received from the surgeon, prior to *in vitro* cultivation.

Fourth, as described in the second half of Chapter 4, we directly tested the possibility raised by the Luria-Delbrück analysis, namely that epithelial cells containing hypermethylated *p16* alleles exist *in vivo* in histologically normal breast tissue. In a generous collaboration with the Baylin, Herman, and Nuovo labs, we indeed showed that a sizeable fraction of reduction mammoplasty specimens contain hypermethylated *p16* alleles, strongly suggesting that vHMEC exist *in vivo*.

I finally conclude, in Chapter 5, by presenting a summary of several salient points of my research in the context of the additional new developments in our understanding of the HMEC/vHMEC system. I also speculate about the physiological and/or pathological relevance of our *in vitro* studies to the all-important *in vivo* setting.

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Figure 1-1

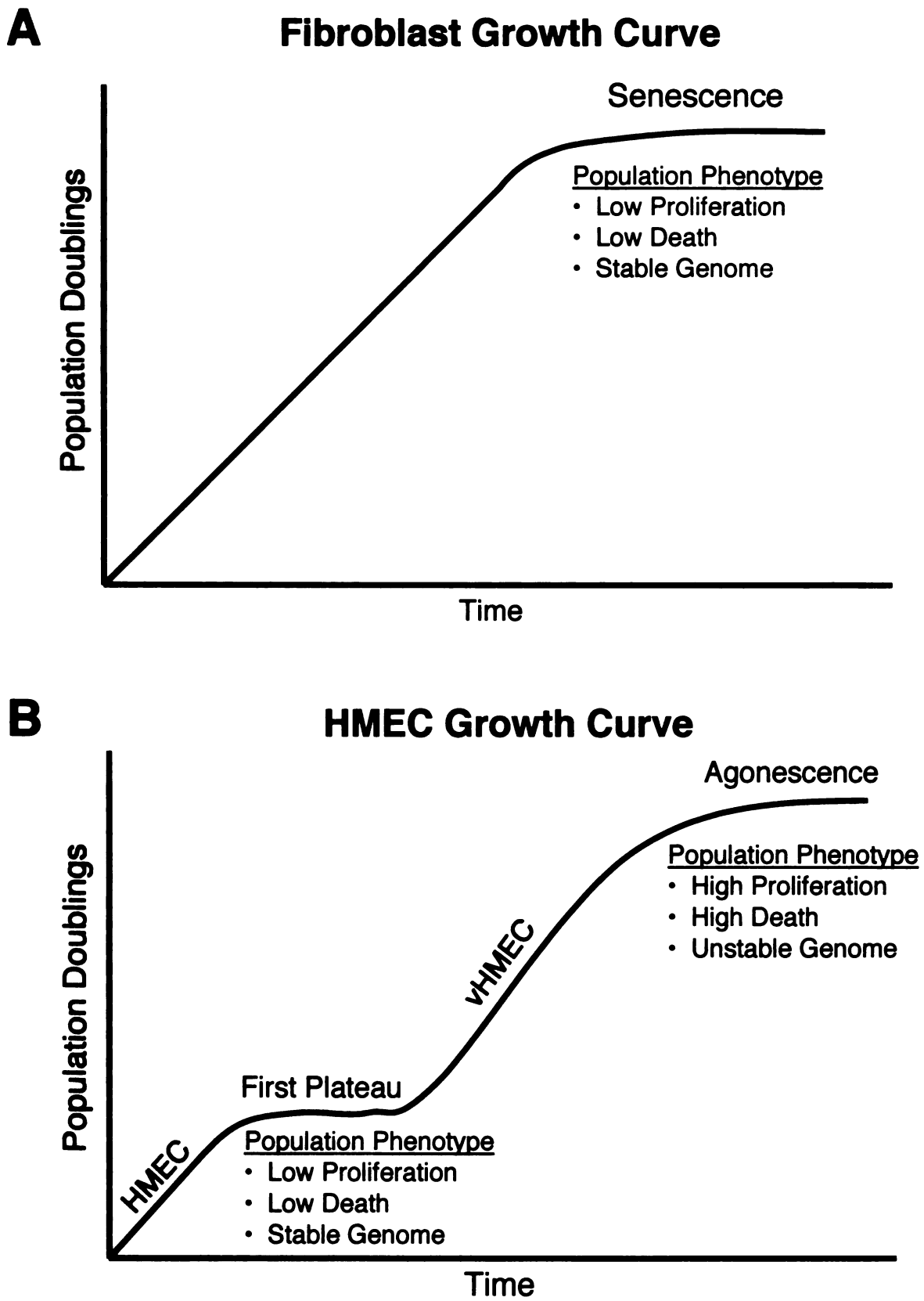
A
Fibroblast Growth Curve
Senescence
Population Phenotype
• Low Proliferation
• Low Death
• Stable Genome
Population Doublings
Time
B
HMEC Growth Curve
Agonescence
Population Phenotype
• High Proliferation
• High Death
• Unstable Genome
vHMEC
First Plateau
Population Phenotype
• Low Proliferation
• Low Death
• Stable Genome
HMEC
Population Doublings
Time

Fig. 1-1. Summary of nomenclature utilized in this document and the phenotypic characterization of population growth plateaus of HMEC and fibroblast cells *in vitro*, as summarized previously ((Romanov et al., 2001) and references therein).

CHAPTER 2

p16 is Necessary for a Senescence-like Population Growth Arrest of Human Mammary Epithelial Cells

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CREDITS

We received the p16 short hairpin RNA construct described herein from Drs. Scott Lowe and Gregory Hannon. Their readiness and willingness to share their valuable reagents prior to their publication exemplifies an admirable scientific cooperation, for which we are very grateful. I performed all of the experiments presented here, with assistance by Jianmin Zhang on the immunocytochemical staining and the Western blot analysis. This work is contained within a manuscript in preparation by J. Zhang, T. Tlsty, and myself.

ABSTRACT

Human mammary epithelial cell (HMEC) populations do not undergo classical replicative senescence (Romanov et al., 2001). Rather, HMEC populations exhibit two phases of exponential proliferation, punctuated by two population growth plateau. The first plateau is phenotypically similar to fibroblast senescence, except that a rare subpopulation of HMEC, called vHMEC here, can spontaneously proliferate beyond the first plateau. The emergence of vHMEC from the first plateau correlates with loss of $p16^{INK4a}$ expression due to promoter hypermethylation (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998). It is not currently known if p16 function is necessary for the first plateau of HMEC *in vitro*. To ask this question, we utilized newly developed RNA interference techniques to inhibit p16 activity. Upon suppressing p16 expression in HMEC, we show here that p16 is indeed necessary for the first population growth plateau. Interestingly, we also found that p16 regulates p53 and Rb levels in HMEC, but not isogenic mammary fibroblasts.

INTRODUCTION

A rare subpopulation of variant human mammary epithelial cells (vHMEC) is able to proliferate beyond a senescence-like, telomere-independent, p16-associated *in vitro* proliferative arrest referred to here as the first population growth plateau (Romanov et al., 2001; Tlsty et al., 2001) (Note: the Romanov reference is included as an Appendix to this dissertation). This variant HMEC (vHMEC) population possesses an extended lifespan (an additional 20-70 population doublings) when compared to the remainder of the HMEC population. vHMEC, however, still constitute a mortal cell population, in that the population does not continue to increase in size indefinitely. Rather, vHMEC finally cease *in vitro* population growth at a plateau termed agonescence (Tlsty et al., 2001). Agonescent vHMEC populations are characterized by abundant genomic instability, an unprecedented phenotype for "normal" human cells grown *in vitro* without exogenous treatment with transforming viruses, forced expression of oncogenes, deliberate DNA damage, *etc.* This spontaneous chromosomal instability is reminiscent of the numerous and omnipresent aberrancies observed in pre-malignant and malignant breast lesions *in vivo* (Shen et al., 2000; Teixeira et al., 2002). Primarily for this reason, we hypothesized that this *in vitro* population of cells (vHMEC) represents a pre-malignant cell population (Tlsty et al., 2001). Indeed, continuing experiments in our laboratory have shown that the vHMEC derived from healthy, disease-free, "normal" women possess additional properties typically associated with pre-malignant and malignant cells (Y. Crawford, J. Zhang, K. McDermott, M. Gauthier, A. Joubel, K. Mantei, C. Pickering, and T. Tlsty, personal communication). These additional properties will be discussed in more detail in Chapter Five.

Several lines of evidence implicate the tumor suppressor gene, $p16^{INK4a}$, as playing a functional role in the first plateau (also referred to as selection) of HMEC cultured under standard conditions. First, expression of high-risk human papilloma virus (HPV) E7 oncoprotein is sufficient to abrogate the selection plateau (Foster and Galloway, 1996). Second, increased expression of the p16 protein and associated decrease in cyclin D1-CDK4 kinase activity accompany the plateau(Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998; Wong et al., 1999). Third, vHMEC, the rare subpopulation of HMEC capable of proliferating beyond the first plateau, exhibit hypermethylated-mediated silencing of *p16* expression (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998; Wong et al., 1999). Treatment of vHMEC with a demethylating agent such as 5-deoxyazacytidine is sufficient to permit re-expression of p16 (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998; Wong et al., 1999). Finally, a recent report demonstrated that overexpression of wild-type Cdk4, a cyclin-dependent kinase target of p16 activity, is sufficient to bypass the HMEC selection plateau (Ramirez et al., 2003).

The aforementioned lines of evidence are strongly suggestive of p16 playing a central functional role in the first population growth plateau of HMEC, but are either correlative or reliant on overexpression of highly pleiotropic genes. For instance, in addition to its well-known ability to bind to and inactivate the function of Rb and other pocket proteins, HPV E7 is also capable of inactivating, among other documented targets, $p21^{CIP1}$, $p27^{KIP1}$, AP-1 family transcription factors such as c-Jun, the Mi2 histone deactylase component, type M2 pyruvate kinase (M2-PK), and the S4 subunit of the 26S proteasome (Antinore et al., 1996; Berezutskaya and Bagchi, 1997; Funk et al., 1997;

Zerfass-Thome et al., 1996; Zwerschke et al., 1999). Such pleiotropic activity of E7 makes interpretation of these experiments difficult. Furthermore, the exhaustive characterization of E7 mutants "specific" for inactivation of one or another class of cellular targets is rarely completed for every known E7 target, casting doubt on the "specificity" of these mutants. E7 proteins with point mutations or small deletions are routinely assumed to be loss-of-function mutations, but gain-of-function activities remain a distinct possibility. Although the use of viral oncoproteins as experimental tools has been of tremendous use historically and affords the opportunity to study a relevant etiological agent of cancer, the pleiotropy of viral proteins unfortunately limits their use as "genetic" agents.

vHMEC populations have been molecularly characterized primarily by their inability to express the *p16* gene product due to promoter methylation. However, it is unknown whether loss of *p16* expression alone is sufficient for proliferation beyond the population growth plateau, let alone the newly characterized phenotypic changes of vHMEC. If p16 loss alone is indeed sufficient for proliferation beyond the plateau, then the additional phenotypic changes associated with the vHMEC population could simply be processes normally dependent on p16 activity in HMEC. In simplistic terms, under this model, p16-null HMEC would be the equivalent of vHMEC, and hence would phenocopy vHMEC in all phenotypes examined. Alternatively, even if the growth plateau is p16-dependent, phenotypic changes emergent in vHMEC populations could be p16-independent, implying additional, currently uncharacterized molecular changes of vHMEC.

To determine if p16 is necessary for the first plateau of HMEC, we utilized RNA interference techniques to disrupt p16 activity therein. We furthermore used this tool to address the role of p16 in regulating known vHMEC gene expression changes.

Cells and cell culture. Isolation of HMEC has been described (Hammond et al., 1984). HMEC were cultured in modified MCDB 170 (MEGM, BioWhittaker, USA). HMEC from the following reduction mammoplasty samples were analyzed: RM9, RM20, RM21 (organoids derived in the laboratory of T.D.T.). HMF were isolated and cultured as per routine protocol. HMF were cultured in RPMI 1640 (CellGro) supplemented with 10% FCS (HyClone) and L-glutamine. HMF from reduction mammoplasty samples 48, RM9, and RM21 were studied. Population doublings were calculated using the equation, PD = log(*A*/*B*)/log2, where *A* is the number of cells collected and *B* is the number of cells plated initially.

RNA Interference. The retroviral construct encoding the p16-specific short hairpin RNA (p16 shRNA) under the control of the U6 promoter was generously provided by G. Hannon and S. Lowe (Cold Spring Harbor Laboratories). The shRNA was designed essentially as described previously (Paddison and Hannon, 2002). The p16 shRNA encodes inverted repeats of 27 bp corresponding to nt 381-407 of the human *CDKN2A* cDNA (GenBank Acc. No. NM000077), separated by an 8-nt spacer. The p16 shRNA sequence, whose expression is directed by the activity of the U6 Pol III promoter, was cloned into the *Hpa*I site of the pMSCVpuro retroviral vector (Clontech). Amphotropic retrovirus was produced by transfecting Phoenix-A packaging cells using Lipofectamine PLUS reagent (Invitrogen) or Effectene reagent (Qiagen); 48-72 h post-transfection, virus-containing culture medium was harvested and filtered through 0.45 μm syringe filters. Transfection frequency of Phoenix-A cells was routinely greater than

60%, as determined by fluorescence microscopic observations of cells transfected in parallel with a plasmid containing a GFP expression cassette (data not shown). HMEC were infected twice by exposing them to virus-containing medium, in the presence of 4 μg/ml Polybrene (Sigma), for 4-6 h each with an intervening 20 h recovery period. HMF were infected similarly, except that HMF were exposed to virus-containing medium twice, for 24 h each time. 72-96 h after the first infection, cells were trypsinized and replated in the presence of 2-4 μg/ml puromycin (Sigma). Following infection, cells were maintained constantly in puromycin-containing medium. Infection frequencies of HMEC were routinely in the range of 3-10%, as determined by colony formation assays (data not shown). Infection frequencies of HMF were ~50% (data not shown).

Western Analysis. Cells were lysed in SDS lysis buffer (2% SDS, 150 mM NaCl) supplemented with 1x Complete protease inhibitors (Boehringer Mannheim). Lysates were stored at –80°C until ready to analyze. Protein concentration was estimated using the BCA method (Pierce), after which samples were prepared using standard conditions for SDS-PAGE. For HMEC samples, 5 μg of total cellular protein was fractionated in gradient (4%-20%) polyacrylamide mini-gels (BioWhittaker Molecular Applications) and transferred to Hybond-P (Amersham) membranes. Similar methods were used to separate HMF proteins, except that 20 μg protein was loaded to facilitate detection of the low-abundance p53 protein. Blocking was accomplished by incubation in TBS-T (20 mM Tris, pH 7.5; 137 mM NaCl; 0.1% Tween-20) containing 5% nonfat dry milk. Proteins were detected with antibodies specific for $p16^{ink4a}$ (NeoMarkers, AB-1), p53 (DO1, Santa Cruz Biotechnologies), and β-actin (Sigma). HRP-conjugated goat-anti-

mouse antibody (Gibco) was used as a secondary antibody. Detection was achieved by employing the SuperSignal West Pico chemiluminescence detection protocol (Pierce).

Immunocytochemistry. Cells were grown upon glass coverslips in standard 12- or 24-well tissue culture plates. Cells were fixed in 4% PFA in PBS for 15 min at room temperature and were stored in 0.1% PFA in PBS at 4°C. Cells were further permeabilized using 0.5% Tween-20 (Sigma) in PBS for 5 min at room temperature. Cells were blocked with 4% goat serum (Sigma) in PBS prior to staining. p16 was detected utilizing the Ab-1 mouse monoclonal antibody (clone DCS-50.1/A7, NeoMarkers). Microscopic images were acquired by CCD camera attached to a Nikon TE300 epifluorescence microscope.

Results and Discussion

We sought to test directly whether p16 is necessary for the growth arrest of HMEC. To ask this question, we utilized recently developed RNA interference (RNAi) techniques to interfere with p16 expression specifically in primary human mammary epithelial cell cultures. RNAi is an experimental tool capable of abrogating gene function in a highly sequence-specific and effective manner (Paddison and Hannon, 2002). RNAi takes advantage of evolutionarily conserved mechanisms to selectively degrade cognate mRNA transcripts identical to short (18-29 nt) double-stranded RNA (dsRNA) fragments. Such short dsRNA molecules can be generated by endogenous Dicer enzymes from longer dsRNA templates, by a variety of *in vitro* preparation methods, or, as in the approach we undertook, by the transgenic expression of a short hairpin RNA molecule. We specifically used pMSCV-puro retrovirus-mediated delivery of a transgene encoding a 27 nt double-stranded hairpin under the control of the U6 Polymerase III promoter (Fig. 2-1, *A*). The short hairpin RNA (shRNA) was designed to target the *p16* exon 1α transcript region (nt 381-407). We were careful to target a region of the *p16* transcript that did not share identity with the $p14^{ARF}$ transcript. Because vertebrate cells lack the ability for dsRNA fragments to "spread" along the target transcript, we predict that our experimental approach should affect *p16* expression, but not $p14^{ARF}$ expression. We tested the effectiveness of the p16 shRNA to suppress gene expression by infecting D98 cells (which express p16 abundantly), selecting for puromycin-resistant transductants, and then assessing gene expression levels by Western blotting and immunocytochemical detection. p16 protein levels were routinely reduced by ~50% in D98 cells 3-5 days post-infection,

when compared to protein levels in cells infected with MSCV lacking the shRNA insert (Fig. 2-1, *B*), thereby providing evidence for the activity of the hairpin.

To test the role of p16 in effecting the HMEC selection growth arrest, we infected HMEC populations within 5 population doublings (PDs) after isolation from primary organoid cultures. Quantitative Western analysis revealed that, similar to the suppressive activity observed in D98 cells, infection by MSCV encoding the p16 shRNA resulted in a 30-70% reduction in protein expression by 5 days post-infection (*e.g.*, Fig. 2-1, *B*), with variability in this effectiveness observed from experiment to experiment. Within 7 days after infection, morphological changes were apparent between vector-infected and p16 shRNA-expressing HMEC. Whereas the vector-infected and non-infected HMEC populations eventually underwent the stereotypical morphological changes associated with the first growth plateau (Fig. 2-1, *D*, and data not shown; note flattened cell shape, increased cell size, and abundant vacuolization), the populations expressing the p16 shRNA consisted of a heterogenous mixture of small, proliferative cells and large, flattened, and apparently non-proliferative cells (Fig. 2-1, *D*). Quantitative immunocytochemical analysis of p16 expression in p16 shRNA-expressing HMEC revealed that, although the mean immunofluorescence intensity decreased by ~60% in the presence of the hairpin, some of the large and vacuolated cells still retained abundant p16 expression (Fig. 2-1, *C*; Fig. 2-2, *A-C*). p16 expression heterogeneity either indicates an inability of the hairpin to suppress p16 expression equivalently in every cell of the population or, alternatively, the hairpin may not be expressed equivalently in all cells. Perhaps the p16 expression heterogeneity explains the phenotypic heterogeneity of the population.

Although the p16 shRNA-expressing HMEC retained morphological heterogeneity for ~7 PD after infection, the population continued to increase in cell number at a rate roughly comparable to that of early-passage pre-selection HMEC (Fig. 2-3). Gradually, the shRNA-expressing HMEC population became more uniformly homogenous in size, which correlated with a continued decrease in p16 protein expression (data not shown). The continued proliferation of the p16 shRNA-expressing HMEC occurred at a time when the non-infected and vector-infected populations were arrested at the first plateau for ~3 weeks, during which time patches of proliferative small cells eventually came to predominate the cultures and overall population growth became apparent again (Fig. 2-3). Similar results were observed in three out of four independent infections of HMEC populations isolated from reduction mammoplasty samples from three different individuals. In one out of four experiments, HMEC infected with the p16 shRNA-encoding retrovirus exhibited proliferation characteristics comparable to the vector-infected HMEC. These results may indicate the presence of additional p16-independent determinants of the selection plateau, a proposal currently under investigation.

In addition to their lack of p16 expression, vHMEC also exhibit other commonly observed gene expression changes when compared to their isogenic HMEC counterparts. In particular, vHMEC express p53 and its transcriptional target p21 at elevated levels relative to HMEC (Romanov et al., 2001). The elevated p53 protein content of vHMEC is likely due to the reported increased protein half-life in vHMEC (3-4 h) relative to values reported in foreskin fibroblasts (0.25-0.5 h) (Delmolino et al., 1993). In addition, vHMEC also express higher protein levels of Rb than isogenic HMEC counterparts. To test

whether p16 expression status specifically affects p53 and Rb gene expression, we assessed their expression in non-infected (Parental), vector-infected, and p16 shRNA-expressing HMEC. Interestingly, the suppression of p16 expression by p16 shRNA expression in HMEC is sufficient to elevate p53 and Rb protein levels (Fig. 2-4, *A*, and data not shown). Conversely, ectopic overexpression of wild-type p16 in vHMEC results in down-regulation of p53 and Rb (J. Zhang and T. Tlsty, personal communication). These data demonstrate an intimate connection between the expression levels and activities of these two important tumor suppression pathways in HMEC. The nature of the interaction between the two pathways in HMEC is unknown, though $p14^{ARF}$ remains a prime candidate as the functional link, as hypothesized by Vousden and others (Bates et al., 1998).

To test whether the interconnectedness between the p16/Rb and p53 pathways is specific to the mammary epithelial cell system, we expressed the p16 shRNA in isogenic human mammary fibroblasts (HMF). Retroviral infection of fibroblasts was efficient, resulting in at least 50% of the target cells being infected. Although the absolute p16 protein content was slightly lower in HMF than in HMEC nearing the first plateau, the p16 shRNA was quite effective at knocking down the expression of p16 in HMF (Fig. 2-4, *B*). This indicates that the p16 shRNA is indeed active in the HMF system. We were surprised to observe that the total p53 and Rb protein levels were unchanged in p16 shRNA HMF when compared to vector-infected HMF (Fig. 2-4, *B*). We conclude that the revealed interconnectedness between the p16 and p53 pathways is cell type-specific, at least under the culture conditions tested here. The functional consequences of these

different gene network connections remain to be explored, but the preliminary evidence remains titillating.

These experiments demonstrate directly that p16 is necessary for the HMEC selection growth arrest. Furthermore, modulation of the p16 expression status is sufficient to recapitulate the vHMEC-associated expression changes of p53 and Rb. In other words, p16 negatively regulates the expression levels of p53 and Rb in HMEC, and, in an unexpected development, this regulation is cell type-specific. Other vHMEC-associated gene expression alterations are under continued investigation, as are the cell type specificity issues raised here.

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Figure 2-1

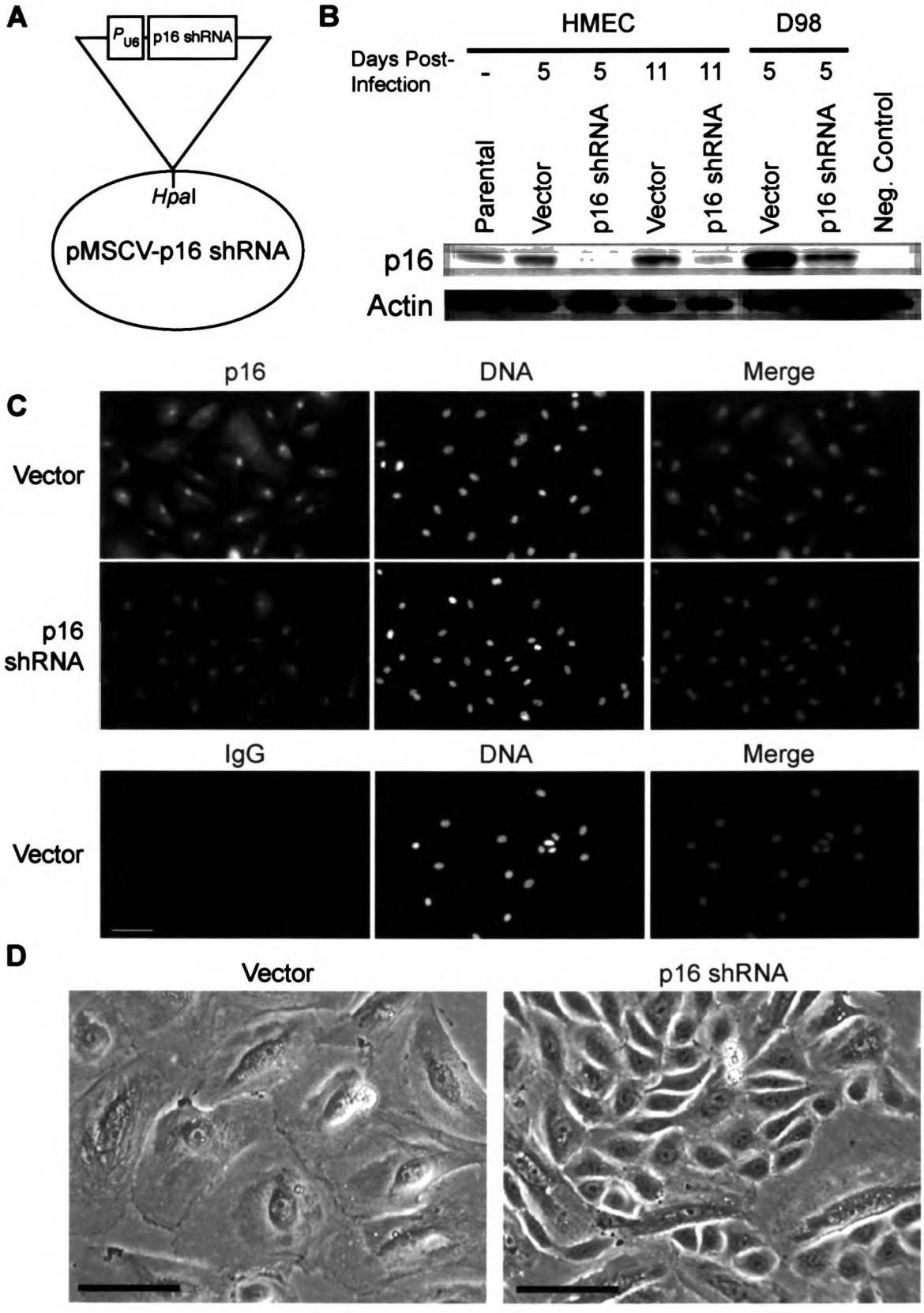
A
P_{U6}
p16 shRNA
HpaI
pMSCV-p16 shRNA
B
HMEC
D98
Days Post-Infection
-
5
5
11
11
5
5
Parental
Vector
p16 shRNA
Vector
p16 shRNA
Vector
p16 shRNA
Neg. Control
p16
Actin
C
p16
DNA
Merge
Vector
p16 shRNA
IgG
DNA
Merge
Vector
D
Vector
p16 shRNA

Fig. 2-1. RNAi effectively down-regulates p16 in HMEC. *A*, Schematic diagram of the retroviral construct utilized in these studies. Expression of the 27-nt p16 short hairpin RNA is under the control of the U6 promoter. The expression of the puromycin resistance gene is controlled by the ubiquitous PGK promoter. Empty-vector consisted of the pMSCV-puro backbone without the U6-shRNA insert. *B*, Western analysis of 5 μg of protein from HMEC or D98 cells, either not infected (Parental), infected by pMSCV-puro vector alone, or infected by pMSCV-p16 shRNA retrovirus. Even loading was determined by β-actin detection. *C, D,* Cellular morphology of vector-infected (*C*) and p16 shRNA-infected (*D*) cells 5 days after infection. Scale bars, 100 μm.

Figure 2-2

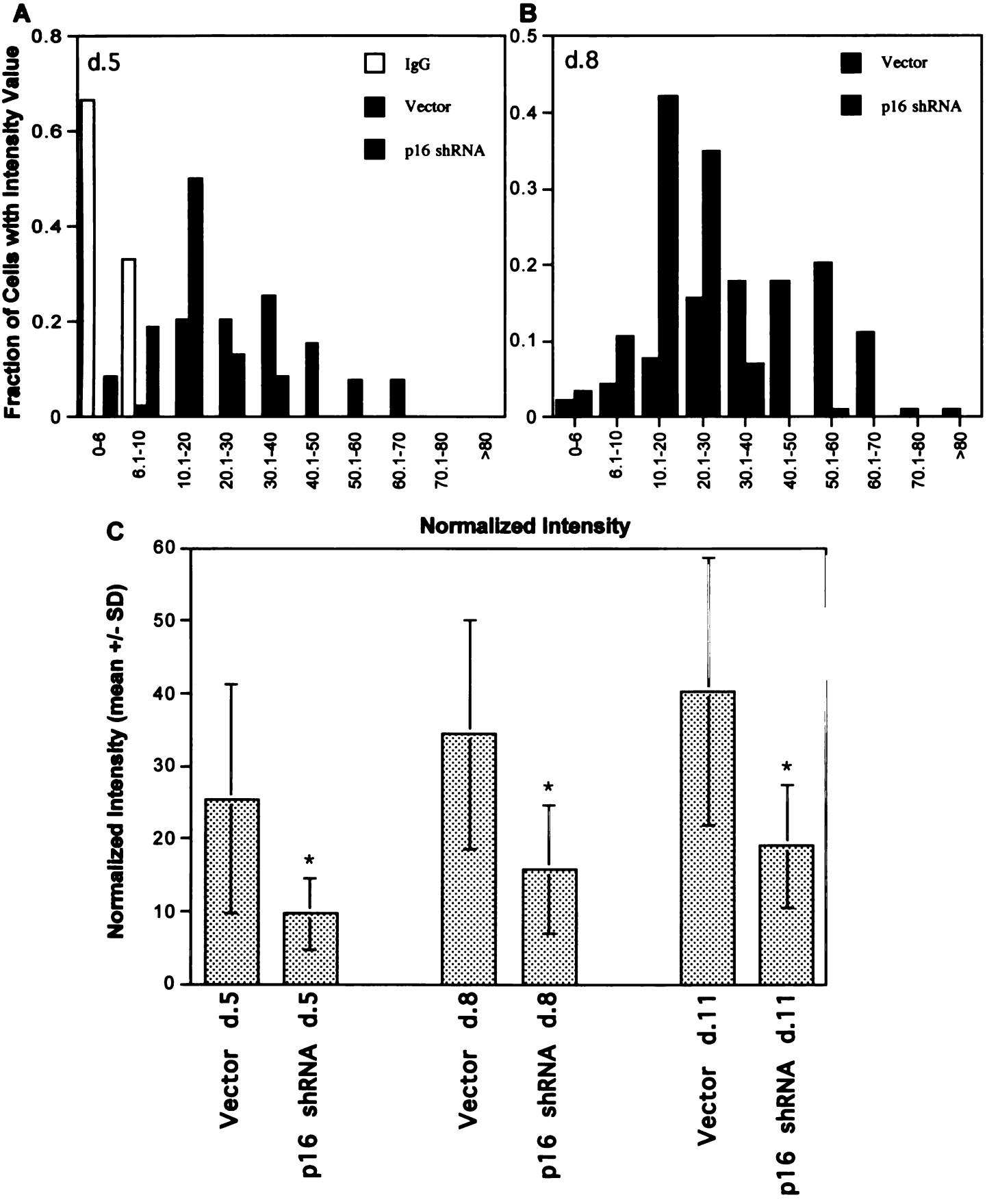
A
d.5
IgG
Vector
p16 shRNA
Fraction of Cells with Intensity Value
0.8
0.6
0.4
0.2
0
0-6
6.1-10
10.1-20
20.1-30
30.1-40
40.1-50
50.1-60
60.1-70
70.1-80
>80
B
d.8
Vector
p16 shRNA
0.5
0.4
0.3
0.2
0.1
0
0-6
6.1-10
10.1-20
20.1-30
30.1-40
40.1-50
50.1-60
60.1-70
70.1-80
>80
C
Normalized Intensity
Normalized Intensity (mean +/- SD)
60
50
40
30
20
10
0
*
*
*
Vector d.5
p16 shRNA d.5
Vector d.8
p16 shRNA d.8
Vector d.11
p16 shRNA d.11

Fig. 2-2. Heterogeneity of p16 shRNA effectiveness. Immunocytochemical detection of p16 was accomplished using standard techniques. Images were acquired with a CCD camera attached to a Nikon epifluorescence microsope, using identical exposure conditions for each image. We then used IP Lab Spectrum to quantitate the average pixel intensity in a standard area contained within each nucleus in several fields of view (as determined by DAPI fluorescence). In addition, the average background intensity was determined in >10 areas in areas of the image not containing any cells. The background intensity value for each independent field of view was subtracted from the p16 staining values for the respective field of view to give the Corrected intensity values. We then summarized the corrected intensity values in the histogram shown. ***, a two-tailed paired t-test showed that $p<0.001$ for each comparison between vector-infected and p16 shRNA-infected HMEC population tested.

Figure 2-3

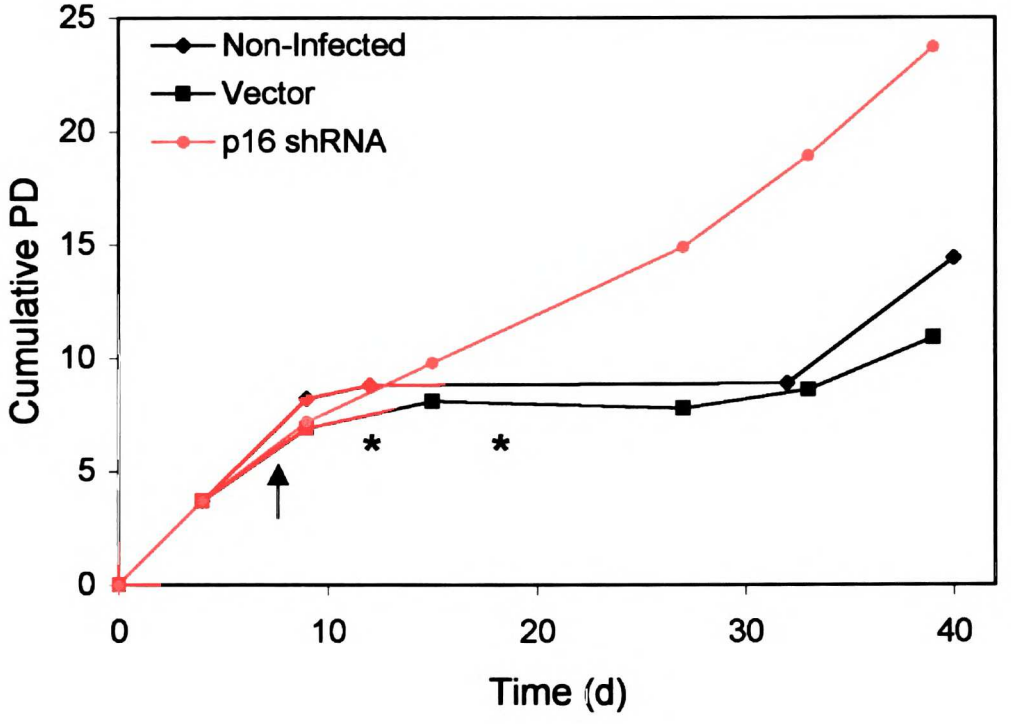
Non-Infected
Vector
p16 shRNA
Cumulative PD
Time (d)
0
5
10
15
20
25
0
10
20
30
40
*
*

Fig. 2-3. p16 is necessary for the first growth arrest of HMEC. Growth curve of HMEC RM9 infected by control virus or p16 shRNA-encoding virus. Arrow indicates time of infection. Puromycin selection occurred 3 d later.

Figure 2-4

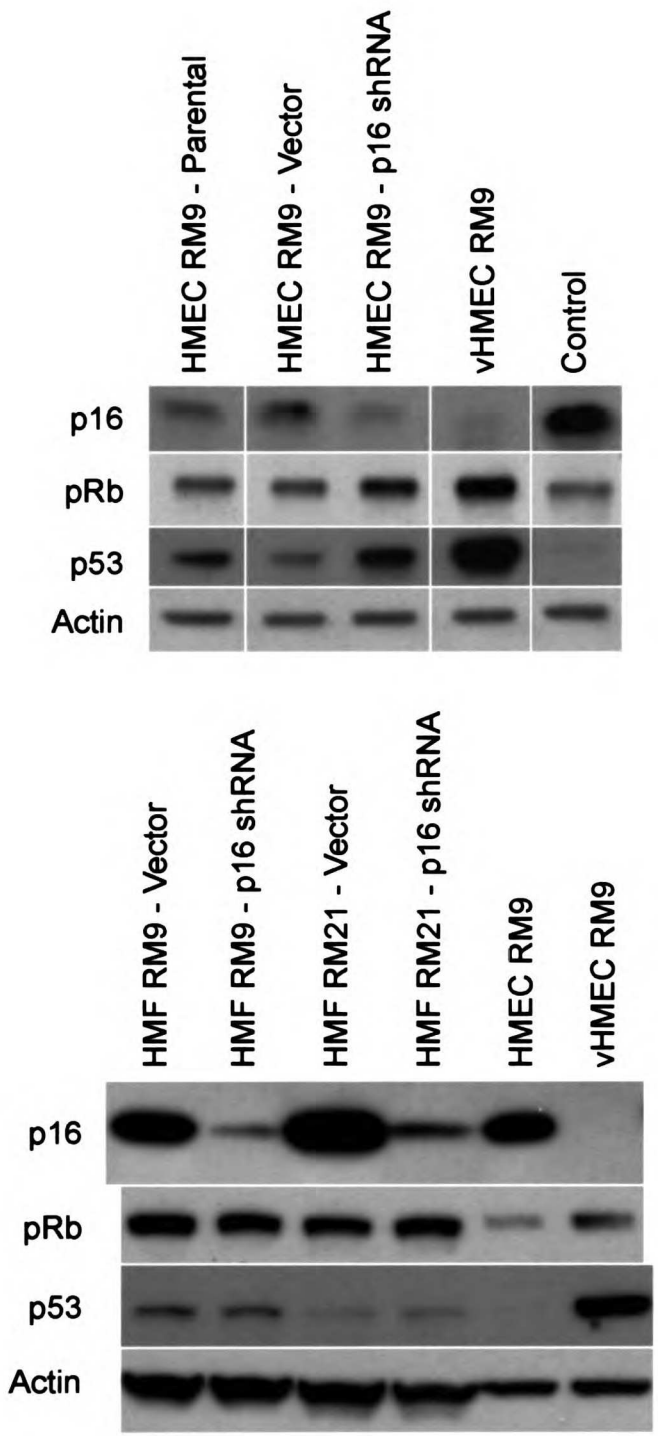
HMEC RM9 - Parental
HMEC RM9 - Vector
HMEC RM9 - p16 shRNA
vHMEC RM9
Control
p16
pRb
p53
Actin
HMF RM9 - Vector
HMF RM9 - p16 shRNA
HMF RM21 - Vector
HMF RM21 - p16 shRNA
HMEC RM9
vHMEC RM9
p16
pRb
p53
Actin

Fig. 2-4. The p16-dependent regulation of p53 and Rb is cell type-specific. Western analysis of HMEC (*A*) and HMF (*B*) expressing the p16 shRNA reveals that p16 down-regulation results in increases in p53 and Rb protein levels in HMEC, but not HMF. vHMEC and D98 (Control) shown in *A* for comparison purposes. In *B*, 5 μg of HMEC and vHMEC total protein were loaded to facilitate comparison of expression levels between the cell types. Note that 20 μg of HMF protein was loaded to allow detection of p53.

CHAPTER 3

Cell type specificity of cell cycle checkpoint control by the p16 tumor suppressor

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CREDITS

As mentioned in the credits to Chapter 2, Dr. Jianmin Zhang and I cooperated to prepare and characterize the HMEC infected by the p16 shRNA provided by Drs. Scott Lowe and Gregory Hannon. Krystyna Kozakiewicz performed all the cytogenetic analyses. Dr. Kimberley McDermott stained and analyzed the colcemid-treated cells for α-tubulin expression. I performed all of the other experiment described herein.

ABSTRACT

Human mammary epithelial cell (HMEC) populations do not undergo classical replicative senescence (Romanov et al., 2001). Rather, HMEC populations exhibit two phases of exponential proliferation, punctuated by two population growth plateaus. The first plateau is defined as a p16-dependent cell cycle arrest, from which a rare, p16-silenced variant HMEC population (vHMEC) arises with low frequency. This distinct vHMEC subpopulation continues to proliferate exponentially in culture until they reach a second plateau, which we previously termed agonescence (Tlsty et al., 2001). Agonescent vHMEC populations are characterized by rampant telomere dysfunction-associated chromosomal instability (Romanov et al., 2001). We hypothesize that the lack of the p16 cell cycle regulator expression in the vHMEC results in loss of cell cycle checkpoint control, thereby permitting genomic instability. In this chapter, we test this possibility and show that, compared to isogenic HMEC and HMF, vHMEC are indeed deficient in two specific cell cycle checkpoints. In response to DNA damage induced by gamma radiation, vHMEC initially arrest in a manner indistinguishable from HMEC and HMF. However, vHMEC fail to maintain this arrest. In response to microtubule disruption, HMEC and HMF exhibit both a 2N and 4N cell cycle arrest; vHMEC are deficient in the 2N arrest. Thus, as suggested before (Meyer et al., 1999), vHMEC have defective cell cycle checkpoint control consistent with a loss in a G1 checkpoint. To test the role of p16 in these two 2N checkpoints, we utilized RNA interference techniques to experimentally suppress p16 function in HMEC and HMF. Surprisingly, we found that (i) the maintenance of DNA damage-induced checkpoint is p16-independent in both HMEC and

HMF, and (ii) the 2N arrest in response to microtubule disruption is p16-dependent in HMEC, but not HMF.

INTRODUCTION

Genomic instability is a hallmark of cancer (Kinzler and Vogelstein, 1998), providing the genetic variability required for tumors to evolve resistance to tumor suppressive safeguard mechanisms present in normal cells. Human mammary epithelial cell (HMEC) cultures derived from the breast tissue of healthy, disease-free women provide a disease-relevant system in which to study the regulation of genomic stability. Observation and experimental manipulation of HMEC *in vitro* has provided a wealth of insights into normal cell biology, as well as possible hints about early changes in breast cancer.

Under standard culture conditions, a distinct subpopulation of cells, to which we refer here as variant HMEC (vHMEC), arise spontaneously in culture at a population growth plateau called either selection or the first plateau. vHMEC are a rare, pre-existing subpopulation of cells that proliferate beyond a *p16*-dependent proliferative barrier that occurs 10-15 population doublings after the explant of HMEC from primary mammary tissue (Hammond et al., 1984; Holst et al., 2003; Romanov et al., 2001; Zhang et al., 2003). vHMEC possess several important characteristics; prominent among these are the absence of *p16* gene expression due to promoter hypermethylation and the eventual acquisition of abundant telomeric dysfunction-associated genomic instability as vHMEC populations approach agonescence. This latter characteristic distinguishes this cell type (under these growth conditions) from isogenic mammary fibroblasts (Romanov et al., 2001) and fibroblasts from other tissues characterized previously.

Unless genetically modified (*e.g.*, by the forced expression of SV40 large T antigen), human fibroblasts at replicative senescence experience a telomere-dependent

cell cycle arrest in the absence of widespread spontaneous karyotypic damage. However, upon the expression of pleiotropic viral oncoproteins such as the E6 and E7 gene products of the high risk human papilloma viruses (HPV), normal human fibroblast populations exhibit profound cell cycle checkpoint defects in response to DNA damage, nucleotide starvation, microtubule disruption, and other cellular insults; proliferate beyond replicative senescence; and, most importantly, accumulate genomic damage spontaneously.

Cell cycle checkpoints serve surveillance roles to ensure correct duplication and segregation of genetic information during the cell division cycle (Weinert, 1998). As has been elegantly demonstrated in *S. cerevisiae*, cell cycle checkpoints function to maintain genomic integrity (Myung et al., 2001; Weinert and Hartwell, 1988). Under certain conditions, cells with cell cycle checkpoint defects may thus become genomically unstable; hence, defective checkpoint control may contribute to the genomic instability observed in cancer cells.

Based upon these observations, we hypothesized that the genomic instability observed in late-passage, agonescent vHMEC populations may be permitted by defective cell cycle checkpoint control in this cell subpopulation. To this end, we compared the cell cycle checkpoint responses to DNA damage induced by gamma radiation and microtubule disruption in vHMEC and the normal HMEC population from which vHMEC arose. We further compared these responses to those of isogenic human mammary fibroblasts. Consistent with our hypothesis, we found that, compared to isogenic HMEC and HMF populations, vHMEC populations exhibit substantial, but specific, checkpoint defects. In particular, vHMEC fail to maintain a long-term arrest in

response to DNA damage caused by gamma radiation. vHMEC also fail to arrest with a 2N DNA content in response to microtubule disruption. Using RNA interference experimental approaches, we showed that the p16 dependence of these cell cycle checkpoints is not only checkpoint-specific (*i.e.*, the microtubule integrity checkpoint is p16-dependent, while the maintenance of the DNA damage checkpoint is p16-independent, in HMEC), but also cell type-specific (*i.e.*, both checkpoints are p16-independent in HMF). These results confirm our hypothesis that vHMEC possess defective cell cycle checkpoint control, and maintain the possibility that such checkpoint deficits might permit the major chromosomal changes associated with vHMEC agonescence.

Cells and cell culture. Cell culture was performed essentially as described in the Methods section of Chapter 2. HMEC from the following samples were analyzed: 184, 48 (provided by M. Stampfer); 1001-3, 4678-2 (commercially available from BioWhittaker); and RM9, RM16, RM20, RM21 (organoids derived in the laboratory of T.D.T.). HMF from reduction mammoplasty samples 48, RM9, and RM21 were studied.

RNA Interference. RNA interference was accomplished as described in Chapter 2. Retrovirally infected HMEC and HMF populations were selected by puromycin addition to the culture medium. Typically, cell cycle experiments were performed 3-10 days after infection.

Cell Cycle Analysis. Cells were metabolically labeled with 10 mM bromodeoxyuridine (BrdU) for 4 hours prior to harvest. Cells were isolated by collecting the culture medium and floating cells therein, followed by standard trypsinization of the adherent cells. After pelleting by centrifugation, the cells were resuspended in PBS and fixed by dropwise addition of ice-cold ethanol until a final concentration of 70% ethanol was attained. Nuclei were isolated and stained with propidium iodide and FITC-conjugated anti-BrdU antibodies (Becton Dickinson, USA), as previously described (White et al., 1994). Flow cytometry was performed on a FACS-Sorter (Becton Dickinson), using CellQuest software for analysis. All analyzed events were gated to remove debris and aggregates.

Cell Sorting. To reduce the hyperdiploid fraction of vHMEC, we utilized the flow cytometric observation that small cells (FSC^{lo}), with low granularity (SSC^{lo}), tended to be enriched for diploid cells (data not shown). We sorted a population of live vHMEC that were FSC^{lo}/SSC^{lo} using a Becton Dickinson fluorescence-activated cell sorter (FACS). This reduced the fraction of cells with a >4N DNA content in an asynchronously dividing population by ~3-fold (data not shown).

DNA damage reduces S-phase entry persistently in HMEC

We sought to investigate the integrity of cell cycle checkpoints in human mammary epithelial cell populations prior to the emergence of the vHMEC variant subpopulation. To accomplish this, we observed the cell cycle distribution of early-passage HMEC populations before and after DNA damage induced by 4 Gy gamma radiation. This dose of radiation is sufficient to cause multiple double-strand DNA breaks per cell in the population (Puck, 1958; Puck et al., 1997). To assess cell cycle distribution, we first pulsed the cell populations with the nucleotide analog bromodeoxyuridine (BrdU) for four hours prior to harvesting, then we isolated cell nuclei and simultaneously stained with propidium iodide to determine DNA content and immunochemically detected cells whose nuclei contained incorporated BrdU. These two parameters were detected simultaneously using flow cytometry, as previously described (White et al., 1994).

Prior to irradiation, 22% of diploid RM21 HMEC incorporated BrdU during the 4-hour pulse (Fig. 3-1, *A*, top left panel), an indication of the fraction of the population synthesizing DNA during that time frame. 69% of the same population contained a 2N DNA content, but were BrdU-negative (presumably cells in the G1 and G0 cell cycle phases). Another 6% of HMEC RM21 contained 4N DNA content and were BrdU-negative (G2 and mitotic cells, but could include a tetraploid G1 population). Finally, ~3% of cells contained >4N DNA content, indicative of a subpopulation of cells with ploidy exceeding diploidy.

To assess the cell cycle checkpoint response of HMEC to gamma radiation, we determined the fraction of cells in various cell cycle phases at different times after irradiation. One day after irradiation, HMEC RM21 reduced the BrdU-positive fraction to 12% (57% of the fraction observed in the initial, untreated population). This change in S-phase fraction was accompanied by no change in the 2N/BrdU-negative (from 68% to 67%) fraction and an increase in the 4N/BrdU-negative fractions (from 6% to 14%). The S-phase fractions further decreased to 5% (25% of control) 2 days after irradiation, and finally leveled at 6% (27% of control) and 5% (25% of control) on days 3 and 5, respectively, post-irradiation. This greatly reduced S-phase fraction is notable because of its persistence. Although a small fraction of cells (~5%) still continued to incorporate BrdU after 4 Gy gamma radiation, the majority arrest with a 2N and 4N DNA content, consistent with activation and persistent maintenance of the G1 and G2 DNA damage checkpoints.

The persistent reduction in DNA synthesis exhibited by HMEC RM21 after gamma radiation was accompanied by a cessation of population growth (data not shown). Indeed, the total cell number ceased to increase the first day after irradiation, and this lack of population growth continued until day 5, the termination point of the experiment. We did not observe an appreciable fraction of cells with <2N DNA content, as would be indicated in a cell population undergoing apoptosis (Fig. 3-1, *A*, and data not shown). We conclude that 4 Gy gamma radiation is sufficient to cause a persistent cell cycle arrest in HMEC.

To test whether the observations in this single experiment were representative of HMEC DNA damage checkpoint responses in general, we repeated the experiment a total

of five times, in HMEC isolated from 3 different individuals. As shown in Figure 3-1, *B*, the trends observed in HMEC RM21 are indeed representative. The relative BrdU-positive fraction (relative to the fraction in the untreated cells on the day of irradiation) reduced to 42±13% (mean±SEM), 25±6%, 25±2%, and 24% on days 1, 2, 3, and 5, respectively. From these experiments, we conclude that within 2 d of damage, the majority of HMEC arrest with a 2N or 4N DNA content. Furthermore, this arrest persists for at least 5 days after irradiation.

Similar to HMEC, HMF arrest stringently in response to DNA damage

We previously reported that human mammary fibroblasts arrest more robustly in response to gamma radiation than do their isogenic vHMEC counterparts (Meyer et al., 1999). We sought to extend the analysis of the DNA damage checkpoint response of HMF to the longer-term observations made here. As is evident in Figure 3-1, *A*, *B*, and consistent with our previous observations (Meyer et al., 1999), at early time-points after DNA damage, HMF populations reduced their S-phase fraction to a greater degree than did either HMEC or vHMEC populations (p=0.057 HMEC vs. HMF, p=0.023 vHMEC vs. HMF, by two-tailed t-test). Similar to HMEC populations, the checkpoint response of HMF was persistent, with DNA synthesis continuing to be inhibited to a large degree even 5 days after damage. Also similar to the HMEC phenotype, HMF populations ceased to increase in total cell number after exposure to a 4 Gy dose of gamma radiation (data not shown).

Isogenic vHMEC initially arrest after DNA damage, but fail to maintain the arrest

As the populations approach the agonescence population growth plateau, variant HMECs accumulate massive amounts of genomic instability. We hypothesize that deficient or "leaky" cell cycle checkpoints in vHMEC contribute to the tolerance and/or persistence of such spontaneous genomic instability. To begin to test this hypothesis, we investigated the competence with which the vHMEC responded to DNA damage induced by gamma radiation, in an experimental setup identical to that described above for the HMEC populations.

The RM21 vHMEC population, which arose at the selection plateau from the HMEC population RM21 characterized in Figure 3-1, *A*, was assessed with regard to its response to the DNA damage induced by gamma radiation. A representative experiment with vHMEC RM21 is shown (Fig. 3-1, *A*, *middle row* of panels). Whereas untreated control vHMEC populations were asynchronously cycling, with 33% of the cells incorporating BrdU during the 4 h pulse, the diploid BrdU-positive fraction decreased to ~6% (18% of control) one day after radiation exposure. This corresponds to greater than a 5-fold decrease relative to the starting fraction. However, 2, 3, and 5 days after damage, the population recovered somewhat, with15% (45% relative to unirradiated control), 14% (43%), and 22% (67%) of the cell populations, respectively, undergoing DNA synthesis during the respective 4-hour pulse. This near-complete recovery of S-phase competence in irradiated vHMEC populations was never observed in HMEC populations.

Consistent with our observations of the cell cycle dynamics of vHMEC after gamma radiation exposure (short-term cell cycle arrest with longer-term recovery), we observed only a transient population growth arrest (1-2 d), followed by rapid recovery to a growth rate comparable to the untreated population (data not shown). This provides

further evidence of the transient nature of the vHMEC arrest in response to DNA damage and provides evidence that the lack of maintenance of the cell cycle checkpoint results in a continued population growth increase.

To determine if the checkpoint response observed in this experiment was unique to vHMEC isolated from this particular individual (RM21), we repeated the experiment 6 times, in cells derived from 5 different individuals. We observed that, similar to the situation observed in RM21, vHMEC populations initially reduce their S-phase fraction in a manner indistinguishable from HMEC populations (*i.e.*, to 43±15% of starting values on day 1). This initial inhibition of S-phase entry is relaxed as time passes, with the S-phase fraction eventually reaching 70±7% of untreated levels by day 5 (Fig. 3-1, *B*). We conclude that vHMEC initiate the DNA damage cell cycle arrest in a manner indistinguishable from HMEC, yet fail to maintain the arrest. This failure to maintain the arrest is manifest in a continued increase in cell number at the population level, and raises the possibility that cells with radiation-induced genomic damage may persist in the population.

To examine this possibility, we passaged vHMEC 5 days after irradiation and allowed them to continue proliferating another 6-7 days, at which time we prepared metaphase spreads for karyotypic analysis. Surprisingly, nearly 20% of the metaphases contained cytogenetically detectable genomic abnormalities, including translocations and broken chromosomes (Table 3-1). Such chromosomal abnormalities were detected at lower frequencies (10%) in mock-irradiated vHMEC cultures at this point in their growth curve. This observation correlates the ability of a large fraction of vHMEC to reenter the

cell cycle after induced DNA damage and the ability of said populations to tolerate genomically aberrant cells within them.

vHMEC also lack a 2N checkpoint in response to microtubule disruption

In addition to the telomeric dysfunction-associated chromosomal instability (*i.e.*, chromosome end-to-end fusions), late-passage vHMEC populations have increasing proportions of cells that are aneuploid and polyploidy (Romanov et al., 2001) (data not shown). Among the possible mechanisms to explain the acquisition of altered ploidy is a defect in mitotic checkpoint control. To assess the integrity of mitotic cell cycle checkpoint arrest, we inhibited microtubule dynamics with a low dose of Colcemid (25 ng/ml), a well-characterized microtubule inhibitor, and observed consequent cell cycle progression using the same flow cytometric means described above. Using indirect immunocytochemical detection of α-tubulin, we showed that microtubules were much less abundant, though not entirely dissociated, at this concentration of Colcemid (data not shown).

To our surprise, in response to Colcemid treatment, HMEC arrested cell cycle progression not only with a 4N DNA content (as would be expected in a mitotic arrest), but also at the 2N stage (Fig. 3-2, *A*, *B*). In 9 independent experiments on HMEC derived from 5 individuals, the fraction of HMEC with a 2N DNA content only decreased 32±6% (relative to the starting 2N fraction) on the second day after Colcemid treatment (Fig. 3-2, *C*). In the representative example depicted in Figure 3-2, *A* and *B*, the 2N/BrdU- fraction decreased from 74% before treatment to 51% after treatment, a 31% reduction relative to the untreated fraction. We also observed in additional experiments that, in response to

Colcemid treatment, (i) the total cell number did not change appreciably for up to four days afterward; (ii) cells with <2N DNA content were not observed by flow cytometry, indicating a low death index; and (iii) cells with >4N DNA content increased slightly 1-2 days afterward, but then leveled thereafter, indicating that HMEC did not re-replicate their DNA upon treatment (data not shown). Furthermore, time-lapse video microscopic analysis of normal human skin fibroblasts revealed that this dose of Colcemid is sufficient to inhibit proper chromosome separation and subsequent cytokinesis at mitosis (data not shown). From these observations, we conclude that HMEC, in addition to the expected 4N (mitotic) arrest, undergo a 2N arrest in response to microtubule disruption. This 2N arrest is not simply due to an insufficient dose of Colcemid to prevent mitotic chromosome segregation and cytokinesis. These arrests are long-term, in that the cell cycle distribution of HMEC after Colcemid did not appreciably change up to four days after initial arrest (Fig. 3-2, *B*, and data not shown). In summary, microtubule disruption inhibits entry into DNA synthesis, as well as mitotic completion, in HMEC.

A similar cell cycle response was observed in HMF: disruption of microtubule stability not only resulted in a 4N arrest, but also an arrest with a pre-S-phase 2N DNA content (*e.g.*, Fig. 3-2, *A*, *B*). On average, by day 2 after Colcemid addition, the 2N/BrdU-fraction of HMF populations decreased 32±8%, relative to untreated 2N fractions (Fig. 3-2, *C*; 8 experiments, from 4 donors). In phenotypic response to microtubule disruption, HMF and HMEC again appear similar in their cell cycle checkpoint response.

In sharp contrast to the 2N and 4N arrest of HMEC and HMF, however, isogenic vHMEC populations arrested with predominantly a 4N arrest, with few (usually <12%) cells retaining a 2N DNA content after Colcemid treatment (*e.g.*, Fig. 3-2, *A*, *B*). Indeed,

2 days after treatment, the average vHMEC population reduced their 2N proportion of cells 82±2% (relative to the starting 2N fraction) after microtubule disruption, a significantly larger decrease than the ~32% relative reduction observed in HMEC and HMF populations (Fig. 3-2, *C*). In short, diploid vHMEC, in contrast to isogenic HMEC and HMF, fail to inhibit S-phase entry upon microtubule disruption.

Each of the HMEC, HMF, and vHMEC populations did also show increases in cells with >4N DNA content after microtubule disruption. The increase was most prominent in the vHMEC populations. We sought to determine if this increase in >4N DNA content was in fact due to reduplication of the genome, or, alternatively, was due to the presence of a background tetraploid population. Repeated experiments showed that the >4N fraction increased during the first 1-2 days after treatment, but then plateaued thereafter (data not shown). This is in contrast to what would be expected if the >4N increase were due to re-replication, in which case the >4N population would continue to increase with time. We thus hypothesize that this increase in >4N cells is indicative of an intact mitotic arrest of a latent background population of tetraploid cells. To validate this hypothesis, we experimentally reduced the fraction of cells with >4N DNA content by isolating cells with low forward scatter (size) and low side scatter (granularity) by fluorescence-activated cell sorting. This sorting reduced the background fraction of untreated cells with >4N greater than three-fold (from 3% to <1%). In this diploid-enriched population, the proportion of cells with >4N DNA content after Colcemid treatment was indeed substantially reduced (data not shown). The sometimes prominent presence of >4N vHMEC after Colcemid treatment was therefore very likely due to a

substantial background of tetraploid cells, and not due to re-replication after microtubule disruption.

From these observations, we conclude that in response to microtubule disruption, (i) vHMEC lacked the 2N checkpoint prior to S-phase and (ii) vHMEC did not re-replicate their genome. These data support the conclusion that vHMEC possess an intact mitotic and/or "tetraploid G1" checkpoint (Andreassen et al., 2001; Lanni and Jacks, 1998), but lack the pre-S-phase 2N checkpoint of their HMEC and HMF counterparts.

Maintenance of the DNA damage cell cycle checkpoint is p16-independent in HMEC and HMF

A prominent molecular difference between HMEC and vHMEC populations is the expression status of the $p16^{INK4a}$ tumor suppressor gene. Whereas HMEC express detectable quantities of p16 protein, with increasing levels until the first proliferative arrest (Romanov et al., 2001), vHMEC that proliferate beyond the proliferative arrest contain hypermethylated *p16* alleles accompanied by silencing of gene expression. Using newly developed short hairpin double-stranded RNA (shRNA) constructs capable of silencing *p16* gene expression, we recently demonstrated that p16 is indeed necessary for the proliferative arrest of HMEC (Chapter 2 and (Zhang et al., 2003)). This observation raises the possibility that phenotypic differences between HMEC and vHMEC may be due simply to the differences in the *p16* status. We sought to address whether the DNA damage checkpoint difference demonstrated here (Fig. 3-1) was due to *p16* gene status. In other words, we asked whether maintenance of the DNA damage cell cycle checkpoint is *p16*-dependent.

To address this question, we compared the DNA damage checkpoint response in parental HMEC populations, and HMEC populations infected either by retrovirus packaging the MSCV empty vector or the p16 shRNA-MSCV retroviral vector, followed by puromycin selection for infected cells. We routinely irradiated cells 6-8 days after infection (3-4 days after puromycin selection). We previously showed that expression of the p16 shRNA in HMEC results in a 50-60% reduction in p16 protein levels (Fig. 2-4, *A*, and Zhang, *et al.*, manuscript in preparation). It is important to note that this experimental treatment is a knock-down experiment, and not a complete knock-out of the cognate gene product.

Partial loss of p16 function by expression of the p16 shRNA did not result in HMEC acquiring the ability to recover S-phase entry after DNA damage (Fig. 3-3, *C*). Similar results were seen in two independent experiments, in HMEC derived from two individuals. The slight apparent differences observed were not statistically significant ($p>0.05$ by paired two-tailed t-test). We conclude that p16 is likely not necessary for maintenance of the DNA damage cell cycle arrest in HMEC. We cannot rule out the possibility that the lack of a phenotypic response may be due to the fact that the p16 shRNA only partially reduces the gene expression level. It does, however, raise the distinct possibility that the lack of maintenance of the DNA damage checkpoint in vHMEC may be due to additional genetic or epigenetic differences between isogenic HMEC and vHMEC populations, a possibility currently under investigation.

To test the role of p16 in the gamma radiation-induced arrest in HMF, we infected HMF with retrovirus containing either the MSCV empty vector alone or the p16 shRNA-pMSCV construct. We confirmed that the p16 shRNA was quite effective at reducing p16

protein level by Western blot analysis. Indeed, p16 protein levels were reduced >10-fold (Fig. 2-4, *B*), possibly indicating a greater activity of the hairpin in HMF than in HMEC. We then assessed the cell cycle checkpoint response of HMF transduced by empty vector alone or the p16 shRNA to 4 Gy gamma radiation. Similar to our observations in HMEC, we observed that both HMF-vector and HMF-p16 shRNA populations arrested with indistinguishable profiles and kinetics (Fig. 3-3, *D*) after DNA damage. These data indicate that the role of p16 in mediating the DNA damage checkpoint response is likely dispensable in two different mammary cell types.

The 2N checkpoint in response to microtubule disruption is p16-dependent in HMEC, but p16-independent in HMF

To test whether the phenotypic difference between vHMEC and HMEC *vis à vis* the microtubule disruption checkpoint was due to the aforementioned p16 status difference, we assessed the Colcemid-induced phenotype of HMEC expressing the p16 shRNA. Similar to those experiments described above, the parental and empty vector-infected HMEC populations arrested not only with 4N DNA content, but also with 2N DNA content. The presence of the 2N arrest is indicated by the 10±3% and 7±3% relative decrease in 2N fraction, respectively, of non-infected and vector-infected HMEC assessed two days after microtubule disruption (Fig. 3-3, *A*). In contrast, HMEC populations expressing the p16 shRNA retained far fewer cells in the 2N cell cycle phase upon Colcemid treatment, reducing the 2N fraction 40±6% relative to the untreated controls (Fig. 3-3, *A*). These observations were made in HMEC infected in 4 independent experiments, from 3 different donors. From these results, we conclude that HMEC

possess a p16-dependent 2N checkpoint sensitive to microtubule integrity, consistent with the known role of p16 in regulating the G1- to S-phase transition (Sherr and McCormick, 2002).

From a phenotypic perspective, HMF and HMEC arrested with similar cell cycle profiles after microtubule disruption. We aimed to assess whether the 2N aspect of the Colcemid-induced arrest was also p16-dependent in HMF. As already described, we generated HMF populations with greatly reduced p16 protein expression levels by infection with p16 shRNA-encoding retroviruses. Vector-infected HMF populations arrested in a manner similar to non-infected populations; namely, vector-infected HMF arrested not only with 4N DNA content, but also with 2N DNA content. Again, this is reflected in the modest reduction (37±9%) in 2N fraction 2 days after microtubule disruption (Fig. 3-3, *B*). In cells expressing the p16 shRNA, we observed no difference in the relative reduction in 2N fraction (39±3% relative reduction). Because p16 level is effectively knocked-down in this population (Fig. 2-4, *B*), we conclude that the 2N arrest caused by microtubule disruption is p16-independent in HMF. These data show that, although HMF and HMEC appear phenotypically similar, the molecular nature of the 2N arrest is different in the two cell types.

In summary, we have shown that HMEC and HMF arrest similarly in response to microtubule disruption and DNA damage induced by gamma radiation. In contrast, vHMEC have defects in cell cycle checkpoint response to these two damaging agents. vHMEC are characterized by a lack of p16 expression due to promoter hypermethylation. We sought to test whether the checkpoint deficiencies in vHMEC were due to the loss of

p16 expression. Using newly developed RNA interference experimental approaches, we showed that the microtubule integrity checkpoint was indeed p16-dependent in HMEC. However, maintenance of the DNA damage checkpoint response in HMEC was apparently p16-independent. These observations suggest strongly that vHMEC possess additional genetic and/or epigenetic alterations beyond simply loss of p16 expression. In contrast to the situation in HMEC, both of the checkpoints characterized here were p16-independent in HMF. This observation, alongside the differential regulation of p53 and Rb gene products, show that the role(s) of p16 vary from cell type to cell type, and raise intriguing questions about epithelial-mesenchymal differences in regulation of tumor suppressor functionality.

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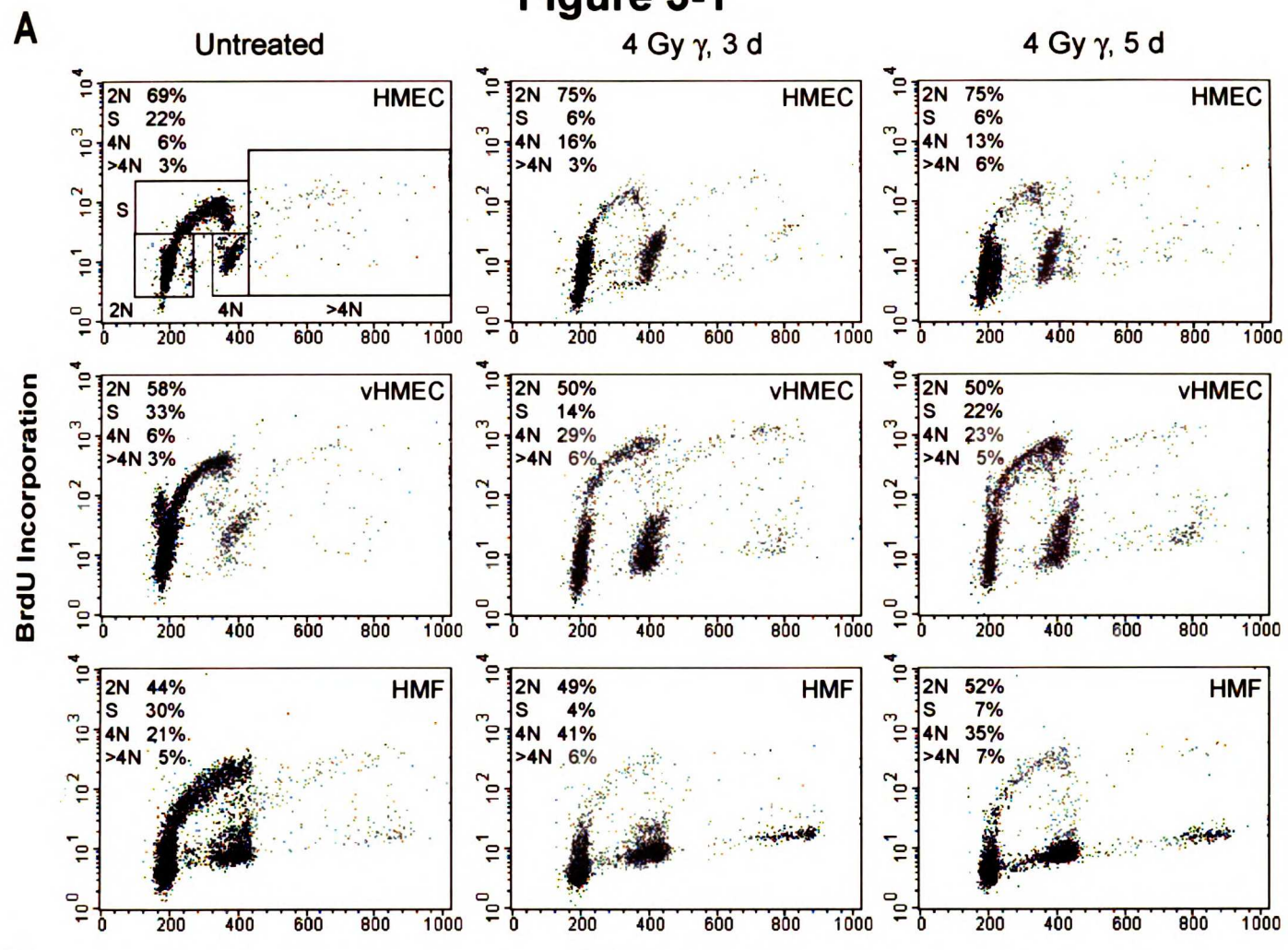
Table 3-1. *vHMEC proliferate in the presence of genomic damage induced by gamma radiation.*

Cell Population	Diploid (46 XX)	Tetraploid (92 XXXX)	Aneuploid	Structural Abnormality
vHMEC RM21 Mock Irradiated	98	0	2	10
vHMEC RM21 4 Gy Gamma	96	0	4	20

50 metaphases were analyzed from each cell population 12 days after irradiation. Numbers indicate the percentage of cells in each category. Definitions used: Aneuploid (any chromosome number beside 46 or 92); Structural Abnormality (includes telomeric associations, deletions, translocations, *etc.*).

Figure 3-1


A
Untreated
4 Gy γ, 3 d
4 Gy γ, 5 d
HMEC
vHMEC
HMF
2N 69%
S 22%
4N 6%
>4N 3%
2N 75%
S 6%
4N 16%
>4N 3%
2N 75%
S 6%
4N 13%
>4N 6%
2N 58%
S 33%
4N 6%
>4N 3%
2N 50%
S 14%
4N 29%
>4N 6%
2N 50%
S 22%
4N 23%
>4N 5%
2N 44%
S 30%
4N 21%
>4N 5%
2N 49%
S 4%
4N 41%
>4N 6%
2N 52%
S 7%
4N 35%
>4N 7%
BrdU Incorporation
DNA Content

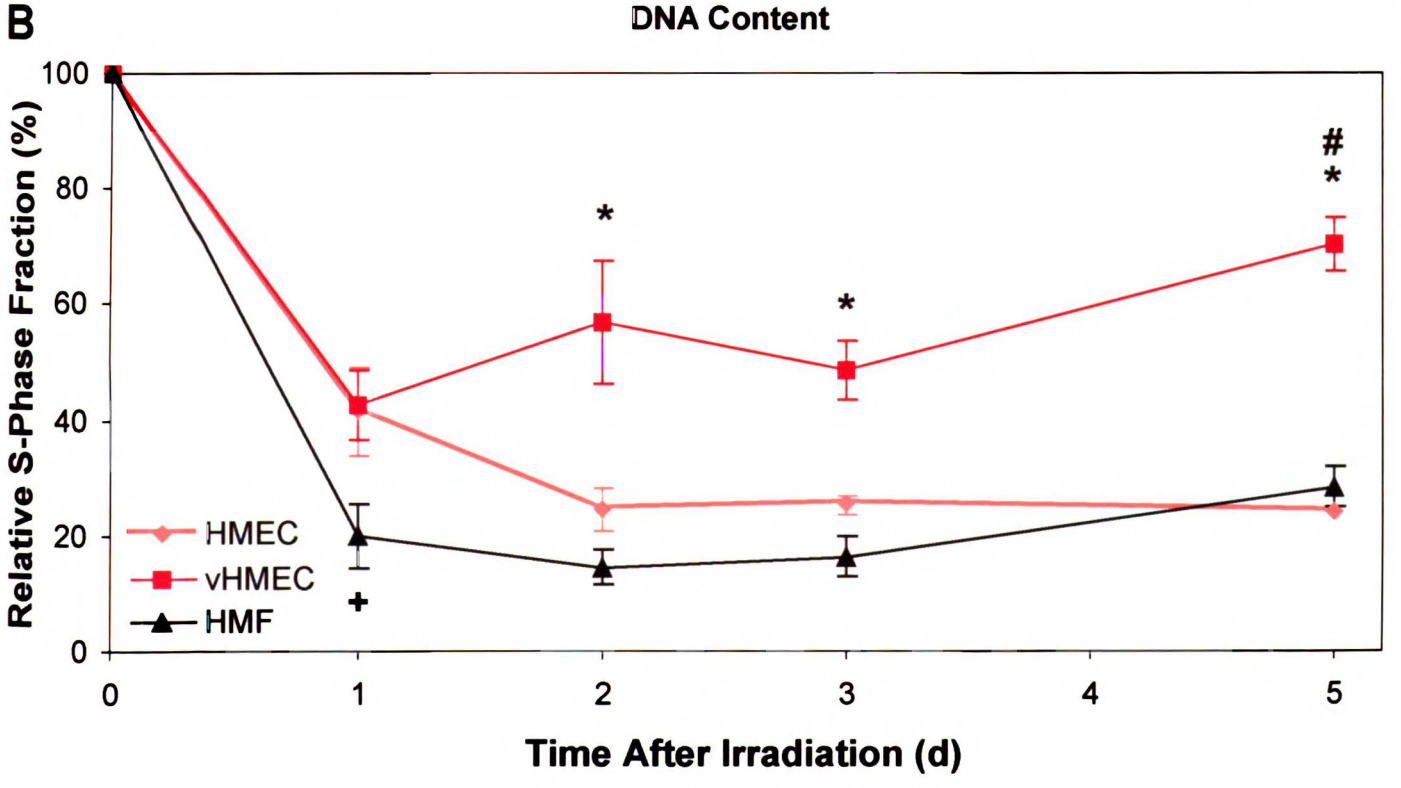
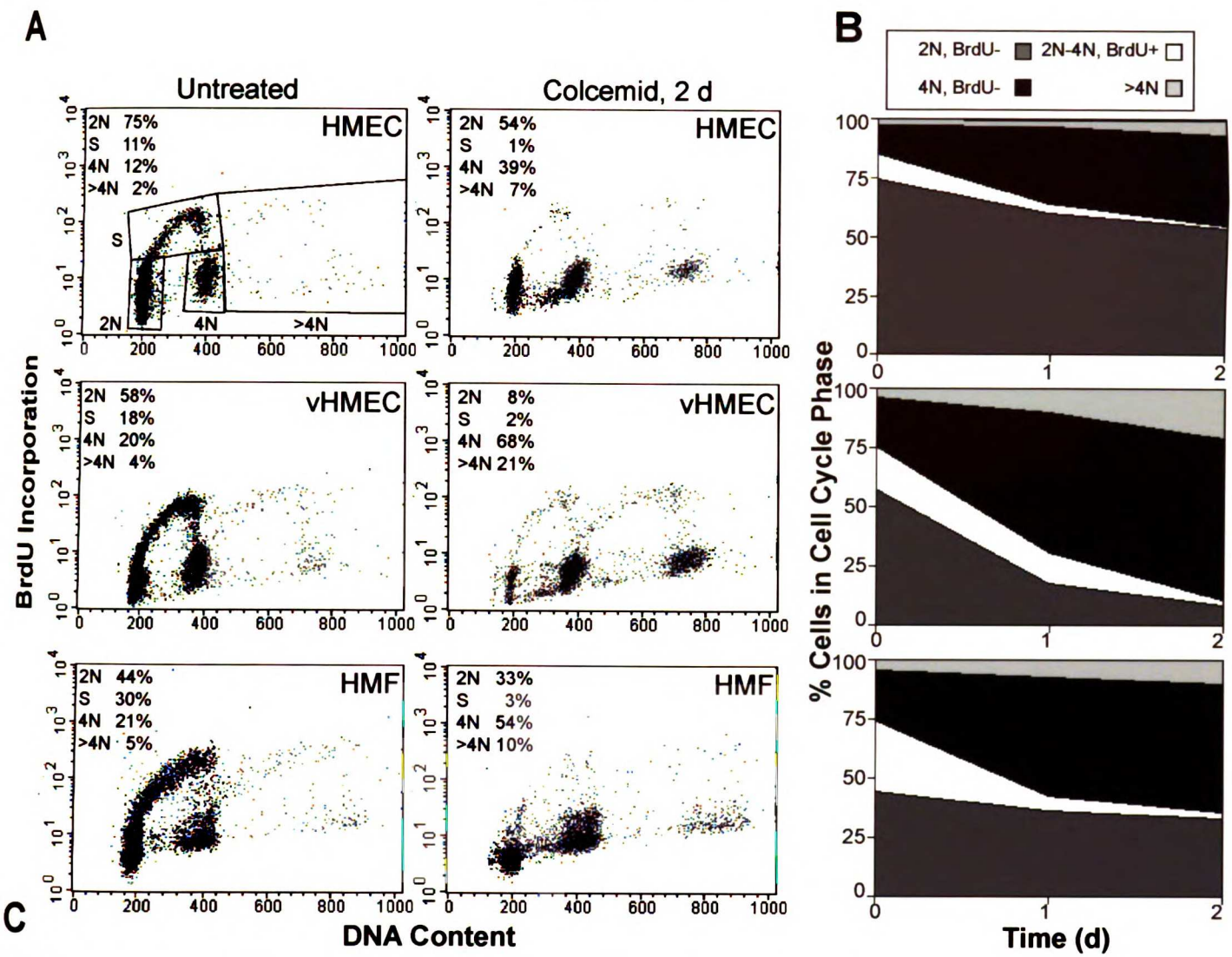

B
Relative S-Phase Fraction (%)
Time After Irradiation (d)
HMEC
vHMEC
HMF
*
*
*
#
+

Fig. 3-1. vHMEC initiate a cell cycle arrest after DNA damage, but fail to maintain the arrest. *A*, Cells were pulsed with BrdU (4 h.), fixed, and stained with anti-BrdU-FITC and propidium iodide, and analyzed using flow cytometry. The *x*-axis represents DNA content as assessed by propidium iodide staining. The *y*-axis represents cells undergoing DNA synthesis, as indicated by positive staining with the anti-BrdU antibody. The rectangles similar to those indicated in the *top left* panel were used to quantify the fraction of cells with respect to their DNA content and BrdU incorporation status. Such quantitation is presented in the *upper left corner* of each panel. Shown, from *left* to *right*, are cells prior to irradiation, 3 d. after 4 Gy gamma radiation exposure, and 5 d. after irradiation. From *top* to *bottom*, representative HMEC, vHMEC, and HMF populations. *B*, Effect of 4 Gy gamma radiation on relative diploid S-phase fraction of HMEC (Pink), vHMEC (Red), and HMF (Black). Diploid S-phase is defined as cells that were BrdU-positive and DNA content of $2N \leq x \leq 4N$. Relative S-phase fraction was defined as the diploid S-phase fraction at time *n* after irradiation divided by the Diploid S-phase fraction in the unirradiated control population at time 0. Tests for significance: *, $p<0.02$ by paired two-tailed t-test between vHMEC and either isogenic HMEC or HMF; +, $p<0.05$, by two-tailed t-test, comparing HMF to either HMEC or vHMEC; #, $p>0.05$ (not significant), by paired two-tailed t-test, when d.5 was compared to d.0 of vHMEC.

Figure 3-2


A
Untreated
Colcemid, 2 d
HMEC
2N 75%
S 11%
4N 12%
>4N 2%
2N 54%
S 1%
4N 39%
>4N 7%
vHMEC
2N 58%
S 18%
4N 20%
>4N 4%
2N 8%
S 2%
4N 68%
>4N 21%
HMF
2N 44%
S 30%
4N 21%
>4N 5%
2N 33%
S 3%
4N 54%
>4N 10%
BrdU Incorporation
DNA Content
B
2N, BrdU-
2N-4N, BrdU+
4N, BrdU-
>4N
% Cells in Cell Cycle Phase
Time (d)

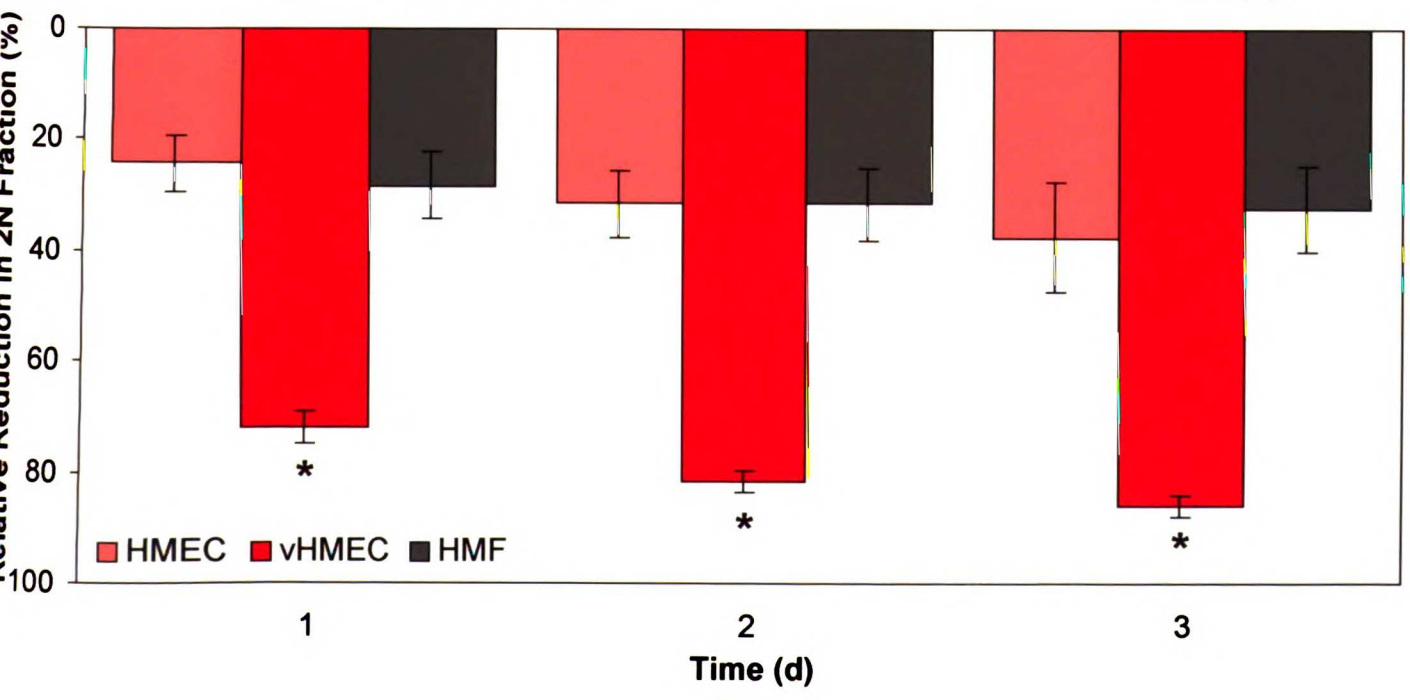

C
Relative Reduction in 2N Fraction (%)
HMEC
vHMEC
HMF
Time (d)

Fig. 3-2. vHMEC also possess a defective 2N checkpoint sensitive to microtubule integrity. *A*, Two-dimensional flow cytometric evaluation of cell cycle distribution either before (*left* panel) or 2 d. after 25 ng/ml Colcemid exposure (*right* panel). Similar quantitation methods to those shown in Fig. 3-1, *A*, were utilized. *B*, The cell cycle data were compiled and presented in the form of an area plot, with the percentage of cells in each phase of the cell cycle (*y*-axis) expressed as a function of time (*x*-axis). *C*, The fraction of cells with a 2N DNA content decreases dramatically in vHMEC after Colcemid addition, whereas the reduction is less pronounced in HMEC and HMF. These data argue that HMEC and HMF possess a 2N, pre-S-phase checkpoint in response to microtubule disruption. Relative reduction in 2N fraction is defined as $(2N_{untreated}-2N_{treated})/2N_{untreated}$. Values for HMEC and HMF were not statistically significant at any time point ($p>0.05$ by unpaired two-tailed t-test). *, vHMEC values were significantly different ($p<0.0001$ by unpaired two-tailed t-test) from both HMEC and HMF.

Figure 3-3

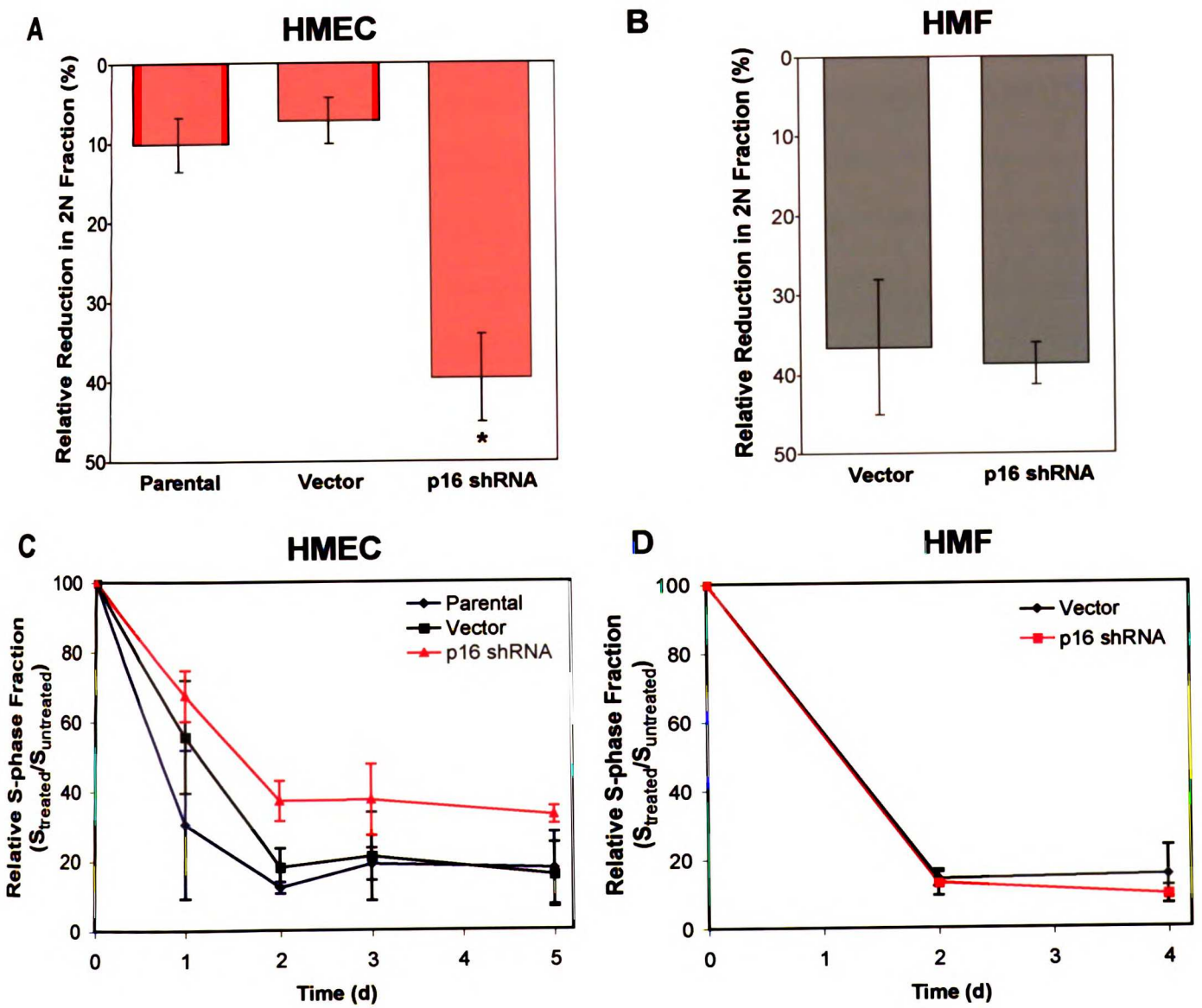

A
HMEC
Relative Reduction in 2N Fraction (%)
0
10
20
30
40
50
*
Parental
Vector
p16 shRNA
B
HMF
Relative Reduction in 2N Fraction (%)
0
10
20
30
40
50
Vector
p16 shRNA
C
HMEC
Relative S-phase Fraction (S_treated/S_untreated)
100
80
60
40
20
0
Parental
Vector
p16 shRNA
0
1
2
3
4
5
Time (d)
D
HMF
Relative S-phase Fraction (S_treated/S_untreated)
100
80
60
40
20
0
Vector
p16 shRNA
0
1
2
3
4
Time (d)

Fig. 3-3. p16 dependence of HMEC and HMF cell cycle checkpoints. *A, B,* Evaluation of the Colcemid-induced cell cycle checkpoint response of mammary cells expressing the p16 shRNA. Cells were assayed as described in Fig. 3-2. The 2N arrest in response to microtubule disruption is p16-dependent in HMEC (*A*), but p16-independent in HMF (*B*). *C, D,* Assessment of the p16-dependence of the DNA damage checkpoint in mammary cells. The inhibition of S-phase entry after DNA damage caused by 4 Gy gamma radiation was p16-independent in both HMEC (*C*) and HMF (*D*). Statistical tests for significance: *, p=0.01 by paired two-tailed t-test, comparing the p16 shRNA-expressing HMEC value to either Parental or Vector-infected HMEC. In panels *B*, *C*, and *D*, p>0.05 by paired two-tailed t-test for all time-matched comparisons.

CHAPTER 4

Methylation of *p16*^{INK4a} Promoters Occurs *In Vivo* in Histologically Normal Human Mammary Epithelia[1]

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RUNNING TITLE *p16* Hypermethylation in Normal Breast Epithelia

KEY WORDS p16^{INK4a}, methylation, breast, epithelial cell, *in vivo*

FOOTNOTES

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[4] The abbreviations used are: HMEC, human mammary epithelial cells; vHMEC, variant HMEC; *p16*, *p16*^{INK4a} (also known as *CDKN2A* and *MTS-1*); MSP, methylation-specific polymerase chain reaction; ISH, *in situ* hybridization; H&E, hematoxylin and eosin; CIS, carcinoma *in situ*; PD, population doubling.

CREDITS

This work is the result of a collaboration between our lab and the laboratories of Drs. James G. Herman and Stephen J. Baylin at The Johns Hopkins University and Dr. Gerard Nuovo at Ohio State University Medical Center. Manel Esteller, James Herman, and Stephen Baylin performed the experiments depicted in Figure 4-2, *A*. Gerard Nuovo did the experiments in Figure 4-2, *B-O*. Under the direction of Karen Chew, the Bay Area Breast SPORE Tissue Core did the immunohistochemical staining in Figure 4-2, *K-L*. I performed the experiments and analyses presented in Figures 4-1 and 4-3, *A-J*, and Tables 4-1 and 4-2. I also analyzed and interpreted the immunohistochemical staining in Figure 4-3, *K-L*.

ABSTRACT

Cultures of human mammary epithelial cells (HMEC[4]) contain a subpopulation of variant cells with the capacity to propagate beyond an *in vitro* proliferation barrier. These variant HMEC, which contain hypermethylated and silenced $p16^{INK4a}$ (*p16*) promoters, eventually accumulate multiple chromosomal changes, many of which are similar to those detected in premalignant and malignant lesions of breast cancer. To determine the origin of these variant HMEC in culture, we used Luria-Delbrück fluctuation analysis and found that variant HMEC exist within the population prior to the proliferation barrier, thereby raising the possibility that variant HMEC exist *in vivo* prior to cultivation. To test this hypothesis, we examined mammary tissue from normal women for evidence of *p16* promoter hypermethylation. Here we show that epithelial cells with methylation of *p16* promoter sequences occur in focal patches of histologically normal mammary tissue of a substantial fraction of healthy, cancer-free women.

INTRODUCTION

HMEC populations do not exhibit classical replicative senescence (Romanov et al., 2001; Tlsty et al., 2001). After 10-15 population doublings in culture, the overwhelming majority of HMEC encounters a proliferation barrier and activates a cell cycle arrest that is phenotypically similar to fibroblast senescence (normal karyotype, low proliferation and death indexes) (Romanov et al., 2001). A subpopulation of HMEC are capable of proliferating beyond this arrest. To avoid possible mechanistic implications, we will, for the purposes of this paper, refer to this cell population as variant HMEC, or vHMEC. At the proliferation arrest, vHMEC appear as colonial outgrowths of small cells among the background of large, vacuolated, non-proliferating cells (Hammond et al., 1984; Romanov et al., 2001). Importantly, vHMEC exhibit several properties that distinguish them from the initial population of explanted cells, including promoter hypermethylation-mediated silencing of *p16* gene expression (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998; Wong et al., 1999). The origin of the vHMEC subpopulation is currently unknown.

Subsequent to the proliferation arrest, vHMEC proliferate an additional 30 – 50 generations beyond the time that the bulk population activates the proliferative arrest. These cells eventually reach a second population growth plateau we previously termed agonescence (Tlsty et al., 2001), which is phenotypically different from human mammary fibroblast senescence and the HMEC first plateau. Agonescent vHMEC populations have both high proliferation and death indexes, although they exhibit no net increase in cell number (Romanov et al., 2001). Furthermore, nearly 100% of vHMEC approaching agonescence exhibit chromosomal defects, including aneuploidy, telomeric associations,

and various other classes of structural abnormalities (Romanov et al., 2001). Such chromosomal instability is reminiscent of the abundant and heterogeneous chromosomal changes observed in pre-malignant and malignant breast cancer lesions (Shen et al., 2000; Teixeira et al., 2002).

To gain insight into the origin of vHMEC, we asked whether this cell subpopulation exists prior to the first population growth plateau. We found that this variant HMEC subpopulation exists before this proliferation barrier. Seeking to extend our *in vitro* observations to the tissue from which HMEC are cultured, we examined histologically normal mammary tissue for *p16* promoter hypermethylation, a defining characteristic of vHMEC. We report here that a significant fraction of normal women contain mammary epithelial cells with *p16* hypermethylation.

Cells and cell culture. Isolation of HMEC has been described (Hammond et al., 1984). HMEC were cultured in modified MCDB 170 (MEGM, BioWhittaker, Walkersville, Maryland), supplemented with isoproterenol (10^{-5} M, Sigma) and transferrin (5 μg/ml, Sigma). We studied HMEC from reduction mammoplasty specimens from four different individuals: 184, 48, 240 (kindly provided by Martha Stampfer, Lawrence Berkeley National Laboratories, Berkeley, California), and RM9 (organoids derived in the laboratory of T.D.T.). Routine cell culture was essentially as described (Romanov et al., 2001), except that cells were seeded at 6.7 x 10^3 cells/cm^2. Population doublings were calculated using the equation, PD = log(*A*/*B*)/log2, where *A* is the number of cells collected and *B* is the number of cells plated initially.

Fluctuation Analysis. Fluctuation analysis experiments (Fig. 4-1, *A*, *B*, *SET* 2) were conducted by (i) imposing a population bottleneck on early-passage HMEC populations, (ii) allowing *in vitro* expansion of the initial populations, subcultivating the cell populations as needed to prevent confluence, until (iii) the cultures ceased increasing in cell number (Romanov et al., 2001). In parallel with SET 2 of samples 184 and 48, mass cultures were propagated (Fig. 4-1, *A*, *B*, *SET 1*) under standard culture conditions. Although *in vitro* propagation of cells, by its very nature, favors cells with a higher proliferation rate, we postulate here that selection for vHMEC occurs predominantly at the first growth plateau. We make this postulation because the proliferation rates and colony forming efficiencies of early-passage HMEC populations and early-passage vHMEC populations are equivalent (data not shown). When the cell populations ceased

expansion, colonies were fixed and stained by standard protocols. Colonies were scored positively if they met the following criteria: colony diameter ≥ 6 mm, staining significantly darker than background, and microscopic confirmation that >90% of the cells in the colony were uniformly small. To validate these colony-scoring criteria, we performed immunocytochemical staining for p16 on representative colonies. Only those colonies that met these criteria were p16-negative (data not shown).

The small subpopulations of founder cells (population bottlenecks) in HMEC 48, 240, and RM9 were derived 8, 10, and 12 days, respectively, after initial seeding of organoids onto the primary tissue culture flask. HMEC 184 populations were derived from second-passage frozen cell populations.

Binomial distributions were calculated according to the formula (using one Poisson assumption, because x<<n):

$$p(x) = f^x (1-f)^{n-x} (n^x/x!)$$

where x is the number of events (colonies), n is the total number of cells plated, and f is the observed frequency. This equation allowed correction for differential cell proliferation from subpopulation to subpopulation. The expected distributions of colony numbers were determined by multiplying the probability distribution by the total number of replicates.

Immunohistochemical Analysis. p16 immunostaining was performed on 5-μm sections of paraffin-embedded tissue using the $p16^{INK4a}$ Ab-4 antibody (clone 16P04, NeoMarkers, Inc., Fremont, CA). Briefly, deparaffinized slides were blocked with 3% hydrogen peroxide, followed by heat-mediated antigen retrieval by microwaving in 10

mM citrate buffer (pH 6.0). Slides were incubated for one hour at room temperature with a 1:200 dilution of the antibody in phosphate buffered saline and 1% bovine serum albumin. Antibody staining was visualized using biotinylated-anti-mouse antibodies (Vector Laboratories, Burlingame, CA) and ABC-HRP Elite (Vector Laboratories, Burlingame, CA), followed by diaminobenzidine reaction. Sections were counterstained with light hematoxylin, and then dehydrated and coverslipped with mounting media.

MSP. The *p16* CpG island methylation status was assessed using a modification of the protocol previously described (Herman et al., 1996). Briefly, DNA was extracted according to standard protocols, denatured by NaOH, modified by sodium bisulfite, purified using Wizard DNA purification resin (Promega), treated again with NaOH, precipitated with ethanol, and resuspended in water. A nested approach was used, first amplifying the bisulfite-modified DNA with the flanking primers 5'-AGA AAG AGG AGG GGT TGG TTG G-3' (upper primer) and 5'-ACR CCC RCA CCT CCT CTA CC -3' (lower primer), "R" being a mixture of A and G. After this step, 4 µl of each 1:1000 diluted flanking PCR reaction was used as a template for methylation-specific PCR, using the primers previously described (Herman et al., 1996). 10 µl of each PCR reaction was loaded onto non-denaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

MSP-ISH. Formalin-fixed, paraffin-embedded tissues (5-µm sections) were used to determine the incidence and cellular distribution of *p16* hypermethylation in clinical samples. MSP-ISH was performed as previously described (Nuovo et al., 1999). After

pepsin digestion of specimens, the DNA was bisulfite modified (CpG Wiz p16 methylation assay; Intergen Discovery Products, Gaithersburg, MD). After a manual hot start (94°C, 3 min), 35 cycles were conducted (55°C, 1.5 min; 94°C, 1 min). PCR utilized the methylation-specific primer set described previously (Herman et al., 1996). After PCR, *in situ* hybridization was performed using a methylated allele-specific internally digoxigenin-labeled probe (1 μg/ml), diluted with Hybrisol VII (Ventana Medical Systems). The amplicon and probe were codenatured (95°C, 5 min), hybridized (37°C, 2 h), washed (1x SSC + 2% BSA, 52°C, 10 min), incubated with anti-digoxigenin alkaline phosphatase conjugate (1:200, Roche Molecular Biochemicals), and then exposed to the chromogen, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Enzo Diagnostics) at 37°C. The final counterstain, nuclear fast red, stains the negative cells pink in contrast to the blue signal. To confirm normal histology, adjacent serial sections were stained with hematoxylin and eosin (H&E), as per standard histological procedures. To confirm staining specificity, adjacent serial sections were treated as above, but omitting the PCR amplification step.

vHMEC exist prior to the first population growth plateau

The chromosomal abnormalities that accumulate in agonescent vHMEC populations are similar to those chromosomal changes present in the earliest lesions of breast cancer. An understanding of the origin of the vHMEC subpopulation of cells is therefore critically important. Specifically, do vHMEC arise *as a result of* the proliferation arrest of the first population growth plateau, *i.e.,* via induction during a stress or adaptation response ("adaptation"); or do they *pre-exist* within the population before the bulk population activates the arrest, and at that time, become *selected* due to a growth advantage ("selection")? We used Luria-Delbrück fluctuation analysis to distinguish between these two models.

Luria-Delbrück fluctuation analysis is a combined experimental and analytical approach that can be used to determine the origin of variant cells that are resistant to a selective pressure (Luria and Delbruck, 1943; Tlsty et al., 1989). Cells are grown under two sets of conditions, SET 1 and SET 2 (Fig. 4-1, *A*), and then analyzed for their ability to generate variants. In SET 1 growth conditions, the cells are aliquoted from a mass culture immediately prior to the selection pressure, thereby measuring the frequency of resistant cells. These aliquots represent a random sampling of cells from the mass population at that time, and the number of variants per aliquot should display a binomial distribution (where, under the condition of a low frequency event, the variance, V, approximates the mean, m).

In SET 2 growth conditions, cells are aliquoted into many replicates of small subpopulations of founder cells (population bottlenecks) and allowed to proliferate for several generations prior to the selection pressure. If the variant cells are generated by conditions found *at* the arrest (adaptation), then previous propagation history should be irrelevant and each subpopulation will have an equal probability of generating variants. The adaptation model predicts that the number of colonies per subpopulation in SET 2 will exhibit a binomial distribution ($V \approx m$). In contrast, under the selection model, if the variant cells *pre-exist* before the selective pressure, then the variability from subpopulation to subpopulation in SET 2 will be higher than that predicted by the binomial distribution ($V > m$).

To apply fluctuation analysis to the question of the origin of vHMEC, we defined the selective pressure as the self-imposed proliferative arrest of the first population plateau, thereby operationally making the first period of exponential proliferation the non-selective period (Fig. 1, *B*). We then measured the frequency and variance of the vHMEC that grew beyond the proliferative arrest. HMEC were cultured according to SET 2 and SET 1 conditions as follows: replicate subpopulations of small numbers of HMEC ($1.0 \times 10^3 - 1.3 \times 10^4$; Table 4-1, SET 2) were cultured separately, while the parental population was cultured in parallel *en masse* (SET 1). Cell populations were allowed to proliferate exponentially (subcultivated as necessary), until the cell number ceased to increase and the cells became large and vacuolated (*i.e.*, they activated the proliferative arrest, data not shown). Cultures were then fed regularly until colonies of p16-negative vHMEC (data not shown) were clearly distinguishable from the background

(14-21 d). At this time, the plates were fixed, stained, and scored for the frequency of cells that could grow beyond the proliferation barrier (Fig. 4-1, *C*).

Fluctuation analyses performed on cell populations derived from four different individuals (HMEC 184, 48, 240, and RM9) indicated that, while the distribution of colonies from the SET 1 populations exhibited the expected binomial distribution ($V \approx m$, Table 4-1), the distribution of colonies among the SET 2 subpopulations did not ($V > m$, Table 4-1, $p < 0.001$ by χ^2 test for each experiment). Similar observations were made in 9 independent experiments, 4 of which are summarized fully here (Table 4-1). These data are consistent with the model that vHMEC are not generated at the time of the proliferative arrest, but rather exist within the population prior to the arrest, and are *selected* at the arrest. For this reason, we argue that the original descriprion of the arrest as "selection" is accurate, and hence this nomenclature should continue to be used (Hammond et al., 1984).

Histologically normal human mammary tissue contains epithelial cells with hypermethylated *p16* promoters

The magnitude of the colony formation frequencies observed in the bottleneck populations of the fluctuation analysis suggested that variant cells not only existed in the population before the proliferative arrest but also were present very early within the culture, and perhaps even *in vivo*. To test the hypothesis that these variant cells exist *in vivo*, we assessed mammary tissue from reduction mammoplasty patients, a patient population with no overt increased risk for breast cancer (Bondeson et al., 1985), for a defining characteristic of post-selection HMEC, namely *p16* promoter hypermethylation

(Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998; Wong et al., 1999). We used the sensitive methylation-specific polymerase chain reaction (MSP) assay (Herman et al., 1996) to ascertain *p16* promoter methylation status in DNA isolated from histologically normal mammary tissue sections. Strikingly, we detected methylated *p16* promoter sequences in DNA isolated from 7 of 15 women (47%, Fig. 4-2, *A*, Table 4-2). All samples that contained methylated-specific PCR product also contained unmethylated-specific PCR product, indicating a mixture of methylated and unmethylated alleles (Fig. 4-2, *A*, and data not shown).

Since the MSP analysis was performed on DNA obtained from an entire histological section, it did not provide information about the cell type that generated the positive PCR signal. To address this issue, we performed methylation-specific PCR *in situ* hybridization (MSP-ISH) (Nuovo et al., 1999) on a partially overlapping set of samples also analyzed by MSP. We detected *p16* promoter methylation in 29% (4/14) of MSP-ISH samples analyzed (*e.g.*, Fig. 4-2, *B*, *C*, *F*, *G*, *J*, *L*; Table 4-2). 71% (10/14) of reduction mammoplasty specimens analyzed by MSP-ISH contained undetectable levels of methylation (*e.g.*, Fig. 4-2, *I*; Table 4-2). Adjacent serial sections were stained with hematoxylin and eosin (H&E) to allow cytological evaluation of the regions of positive staining. Microscopic analysis revealed that methylated *p16* alleles were present in histologically normal mammary epithelial cells in both lobular (Fig. 4-2, *E, F, G*) and ductal (data not shown) regions. Neither myoepithelial cells nor stromal cells (including fibroblasts) contained detectable methylated alleles (Fig. 4-2, *G*). While several of the breast reduction samples were positive by both the MSP and MSP-ISH analyses, 5 samples demonstrated discordance between the two methods of analysis (Table 4-2).

Possible explanations for this discordance include differential sensitivities of the two techniques, the apparent focal nature of the hypermethylated cells, and/or sampling differences.

We then analyzed the distribution pattern and approximate frequency of cells positively staining for *p16* promoter methylation within the tissue and exhibited the data using a tissue map (Fig. 4-3). This method of presenting the data allows a display of spatial information and heterogeneity. In general, the samples fell into 3 major categories. (i) As already mentioned, the majority of samples (10/14) contained an undetectable number of cells per histological section with methylated *p16* promoter sequences (<<1% positive epithelial cells per section; *e.g.*, Fig. 4-2, *H, I*; Fig. 4-3, *A-G,* and data not shown). By calculating the total area occupied by epithelial cells per section and the mean number of epithelial cell nuclei per unit area, we estimate that the average histological section contains ~30,000 epithelial cell nuclei. Thus, in ten of the 14 samples, the frequency of detection is less than 1/30,000, or 3.3×10^{-5}. It is currently unknown whether repeated sampling from different sites of the same breast will reveal similar or different frequencies. (ii) Two samples contained rare foci or an intermittent scattering of cells with methylated *p16* promoter sequences (samples 9624 and 5308; Fig. 4-3, *H*, and data not shown; note frequent juxtaposition of methylated clusters and unmethylated clusters). (iii) Finally, two samples contained a considerable number of cells per section (~10-50% positive epithelial cells per section, though the frequency varied greatly from region to region; samples 9698 and 10811; Fig. 4-3, *I, J*). Large adjoining regions of positivity (for example, as outlined by the green dashed line in Fig. 4-3, *I*) may indicate clonal origin of variant cells or a field effect.

Promoter hypermethylation is frequently associated with silencing of gene expression (Gonzalez-Zulueta et al., 1995; Herman et al., 1995; Merlo et al., 1995). Consistent with this association, we found that regions of *p16* hypermethylation in reduction mammoplasty samples corresponded to regions of low to undetectable p16 protein expression (*e.g.*, Fig. 4-3, *L*, sample 10811; and data not shown). Specimens with undetectable hypermethylation varied in their levels of p16 protein expression; some contained low to undetectable expression levels (data not shown), whereas others expressed p16 abundantly in focal patches (*e.g.*, Fig. 4-3, *K*, sample 10966). The basis for the induction of p16 expression in mammary tissue is currently under investigation.

To confirm the specificity of the MSP-ISH results, an adjacent serial section from each reduction mammoplasty was processed in parallel, but omitting the PCR step. Omission of PCR resulted in loss of nuclear hybridization (Fig. 4-2, *K*, *M*), thereby attesting to the specificity of the post-PCR methodology. Furthermore, with each set of MSP-ISH reactions, we processed a cervical carcinoma *in situ* (CIS) sample shown previously by various methods to contain extensive *p16* promoter hypermethylation, as well as a benign cervical sample previously shown to contain unmethylated promoter sequences (Nuovo et al., 1999). As expected, after MSP-ISH, the cervical CIS sample showed abundant nuclear hybridization (Fig. 4-2, *O*) and signal was undetectable in the benign cervical tissue (Fig. 4-2, *N*). In further control experiments, the CIS sample stained negatively when (i) PCR was omitted, (ii) primers were omitted from the PCR, or (iii) the sodium bisulfite modification reaction was omitted (data not shown).

Discussion

Dysregulation of the p16/cyclin D1/Rb pathway is common to many different cancer types (Chin et al., 1998; Sherr, 1996). Sporadic breast carcinomas frequently exhibit heterozygous or homozygous deletion of the INK4a locus (Cairns et al., 1995), and/or silencing of the *p16* gene by promoter hypermethylation (Esteller et al., 2001). We show here that hypermethylation of the *p16* promoter can occur in morphologically normal mammary epithelial cells from a sizeable fraction of women with no overt increased risk for breast cancer. The clinical relevance of this event in normal women is currently under investigation. It is possible that, in the breast, *p16* hypermethylation serves a "normal," as-yet-undetermined biological function unrelated to carcinogenesis. However, because of the extensive clinical and experimental evidence implicating the *p16* gene as a tumor suppressor, we favor the interpretation that the observed *p16* hypermethylation is a common and early event in sporadic breast cancer. In keeping with the current multistep model of initiation and progression in breast cancer, we posit that additional epigenetic and/or genetic lesions beyond *p16* silencing would be necessary to manifest the malignant phenotype.

If the *p16* promoter-hypermethylated variant epithelial cells indeed represent precursors to breast cancer, our observations suggest that premalignant breast lesions are more frequent than generally appreciated. Studies by Nielsen and colleagues (Nielsen et al., 1987) and Alpers and Wellings (Alpers and Wellings, 1985) have shown a surprising degree of undetected premalignant and malignant lesions. In the Nielsen study of double mastectomy specimens from 110 medicolegal autopsies, whose cause of death was unrelated to breast cancer, nearly one-third of women harbored hyperplastic lesions

(32%), over one-quarter contained atypical ductal hyperplasia (ADH, 27%), almost one-fifth showed ductal carcinoma *in situ* (DCIS, 18%), and 2% had overt invasive breast cancer. Furthermore, almost half of the women with ductal carcinoma *in situ* (DCIS) had bilateral (41%) and/or multifocal (45%) disease (Nielsen et al., 1987). Alpers and Wellings' study of 185 breast samples from random autopsies confirmed this high prevalence of undetected premalignant breast lesions (Alpers and Wellings, 1985). Other studies have reported lower frequencies of pre-malignant lesions (Bartow et al., 1987; Bhathal et al., 1985), but sampling methods and clinical definitions varied among these studies (Welch and Black, 1997). These data indicate that the initiation of premalignant lesions, identified by morphological alterations within the tissue, is by no means a rare event.

Our observation of *p16*-methylated variant cells in histologically normal tissue may be identifying premalignant lesions prior to the morphological changes reported above. Several recent studies have shed light on the genomic status of histologically normal breast tissue. Deng *et al.* (Deng et al., 1996) showed that a common genomic alteration in primary invasive breast cancers (loss of 3p) often occurred in adjacent morphologically normal ductal tissue. Using a broader range of markers, Larsen *et al.* (Larson et al., 1998) showed that 22% of microdissected histologically normal breast samples showed microsatellite instability and/or loss of heterozygosity. Kandel *et al.* (Kandel et al., 2000), furthermore, showed that p53 mutations, including missense mutations previously detected in breast cancer, could be detected in normal and benign breast tissue. These observations, along with the epigenetic alteration reported here, support the hypotheses that early premalignant breast lesions are more frequent, and

harbor more genetic and epigenetic alterations, than previously suspected. We anticipate that further study of vHMEC *in vitro* and *in vivo* will continue to provide insights into early changes in breast cancer.

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Table 4-1. *Fluctuation analysis reveals that vHMEC exist prior to the proliferative arrest*

	Cell Population					
	184		48		240	RM9
Category	SET 1	SET 2	SET 1	SET 2	SET 2	SET 2
Replicates (*n*)	26	19	16	24	15	23
Bottleneck Population Size		1.0×10^4		1.0×10^4	1.3×10^4	1.0×10^3
PD (mean±SD)[a]		2.7±0.4		5.1±0.2	7.0±0.5	7.9±1.1
# cells plated (per replicate) for colony formation	1.0×10^4	$\sim 6.5 \times 10^4$	2.0×10^5	$\sim 3.4 \times 10^5$	$\sim 1.7 \times 10^6$	$\sim 2.4 \times 10^5$
# replicates with *X* colonies:						
X = 0	14	14	11	20	9	20
X = 1	10	1	5	1	2	0
X = 2	1	1	0	1	1	0
X = 3	1	0	0	0	0	0
X = 4	0	0	0	1	1	0
X≥5	0	3[b]	0	1[b]	2[b]	3[b]
# colonies per replicate:						
Range	0-3	0-15	0-1	0-17	0-10	0-51
Mean (*m*)	0.58	1.63	0.31	1.0	1.7	3.5
Variance (*V*)[c]	0.57	15	0.23	12	9.2	125
V/m	**0.99**	**9.1**	**0.73**	**12**	**5.5**	**36**
χ^2	**0.4**	**97.7**	**0.3**	**33.6**	**55.7**	**103**
p[d]	**0.98**	**<0.001**	**0.99**	**<0.001**	**<0.001**	**<0.001**
Frequency (*f*)[e]	5.8×10^{-5}	2.3×10^{-5}	1.6×10^{-6}	2.9×10^{-6}	9.7×10^{-7}	1.1×10^{-5}

[a] Population doublings from bottleneck until growth plateau.
[b] Actual SET 2 colony numbers (≥5) – 184: 5, 8, 15; 48: 17; 240: 7, 10; RM9: 12, 17, 51.
[c] $V = SD^2$
[d] *p*-values were calculated using the χ^2 test (Microsoft Excel), with degrees of freedom (df) as follows: 184, 4; 48, 3; 240, 3; RM9, 4.
[e] Mean frequency for a whole SET.

Table 4-2. p16 *Promoter Methylation Status in Normal Breast Specimens*

Patient Identification	Age (yr)	MSP [a]	MSP-ISH [b]
9698	27	+	+
5308	56	+	+
10811	26	+	+
7643	31	+	-
8285	18	+	-[c]
10434	32	+	-
4508	69	+	-
9624	16	-	+
11755	22	-	-
1514	45	-	ND[d]
10824	46	-	ND
12993	21	-	ND
9468	40	-	ND
8275	33	-	ND
5105	45	-	ND
10966	26	ND	-
12075	30	ND	-
12610	31	ND	-
11139	32	ND	-
11018	25	ND	-[c]
Median Age (yr)	31		
Methylated		31	26.5
Unmethylated		36.5	30.5
Samples Methylated (%)		7/15 (47)	4/14 (29)

[a] MSP scoring: -, unmethylated product but not methylated product detected; +, methylated product detected.
[b] MSP-ISH scoring: -, no positively stained nuclei present in entire section; +, positive nuclei detected.
[c] Few epithelial cells present in section.
[d] ND, not done.

Figure 4-1

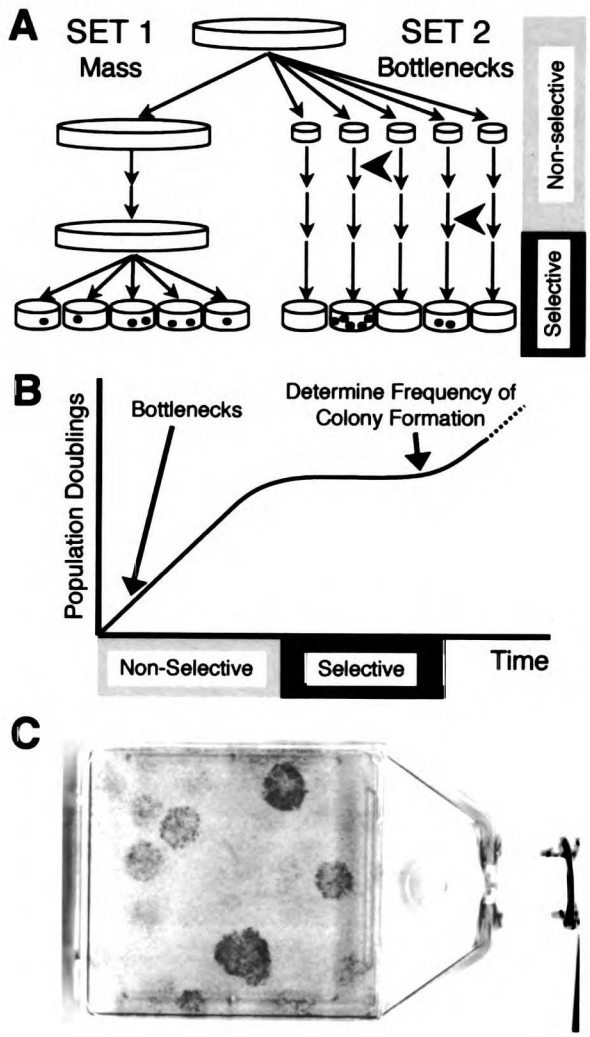
A
SET 1
Mass
SET 2
Bottlenecks
Non-selective
Selective
B
Bottlenecks
Determine Frequency of
Colony Formation
Population Doublings
Non-Selective
Selective
Time
C

Fig. 4-1. Schematic diagrams of fluctuation analyses and representative colonies. *A*, general schematic diagram. SET 1 samples the variance of the mass population; the distribution should be binomial, and the variance should approximate the mean ($V=m$). SET 2 measures the distribution of colony formation in bottleneck populations that have been segregated and propagated before application of the selective pressure. In this example, variants have arisen at times prior to selection (*arrowheads*), making the variance greater than the mean ($V>m$). *black dots,* colonies. *B*, fluctuation analysis experimental design as defined in this study. *C*, colonies of vHMEC in a 25-cm^2 flask stained for scoring.

Figure 4-2

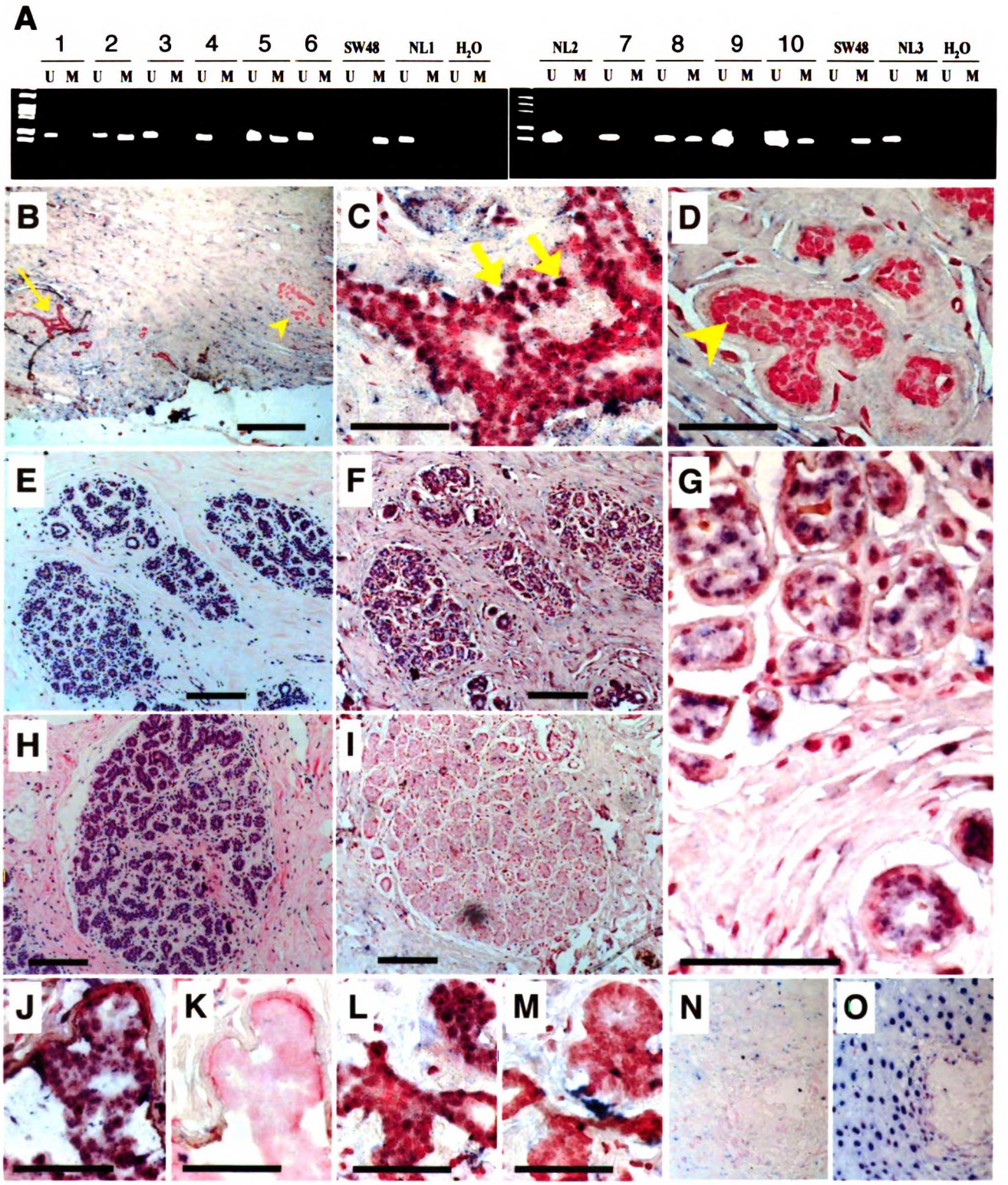
A
1 2 3 4 5 6 SW48 NL1 H_2O
NL2 7 8 9 10 SW48 NL3 H_2O
U M
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Fig. 4-2. Methylation of *p16* in reduction mammoplasty samples. *A*, detection by MSP. The presence of a visible PCR product in those lanes marked "U" indicates the presence of unmethylated alleles; the presence of product in those lanes marked "M" indicates the presence of methylated alleles. The cancer cell line SW48 was used as positive control for methylation, normal lymphocytes (*NL1, NL2* and *NL3*) were used as negative controls for methylation, and water (H_2O) was used as negative PCR control. Sample, patient numbers: *1*, 1514; *2*, 7643; *3*, 10824; *4*, 12993; *5*, 8285; *6*, 9468; *7*, 9624; *8*, 9698; *9*, 11755; *10*, 10434. pBR322/Msp digest is shown *at left* as molecular weight markers. *B-L*, Detection of *p16* promoter hypermethylation in histologically normal mammary epithelia *in situ* by MSP-ISH. *B*, field of view with adjacent positively (*arrow*) and negatively (*arrowhead*) stained regions of epithelial cells (Patient 9698, MSP-ISH). *Blue signal*, hybridization to the MSP product by ISH; *pink signal*, nuclear counterstain. *C*, *D*, higher magnification of the areas indicated in *B* by the arrow and arrowhead, respectively. *E*, *F*, representative lobular epithelial cells with *p16* hypermethylation (Patient 10811; *E*, H&E; *F*, MSP-ISH). *G*, region showing positive epithelial cell staining and negative adjacent stromal fibroblast staining (Patient 10811, MSP-ISH). *H*, *I*, Representative unmethylated lobular epithelial region (Patient 10434; *H*, H&E; *I*, MSP-ISH). *J-O*, Representative examples of MSP-ISH control experiments. Serial sections adjacent to samples positive for *p16* hypermethylation were processed in parallel, with or without the PCR amplification step. This tests the dependency of "positive" *in situ* hybridization results on prior PCR amplification. Shown are representative results for samples from individuals 10811 (*J*, *K*) and 9698 (*L*, *M*). *J*, *L*, representative images of complete MSP-ISH reactions demonstrating *p16* promoter hypermethylation. *K*, *M*, representative images of

control reactions in which the PCR step was omitted. *N, O*, MSP-ISH of negative and positive control samples. In parallel with each MSP-ISH experiment, we processed two samples whose *p16* methylation status was known (Nuovo et al., 1999). *N*, negative control: benign reactive squamous cervical tissue. *O*, positive control: cervical carcinoma *in situ*. *Standard bars*, 460 μm (*B*), 150 μm (*E, F, H, I*), and 45 μm (*C, D, G, J-M*).

Figure 4-3

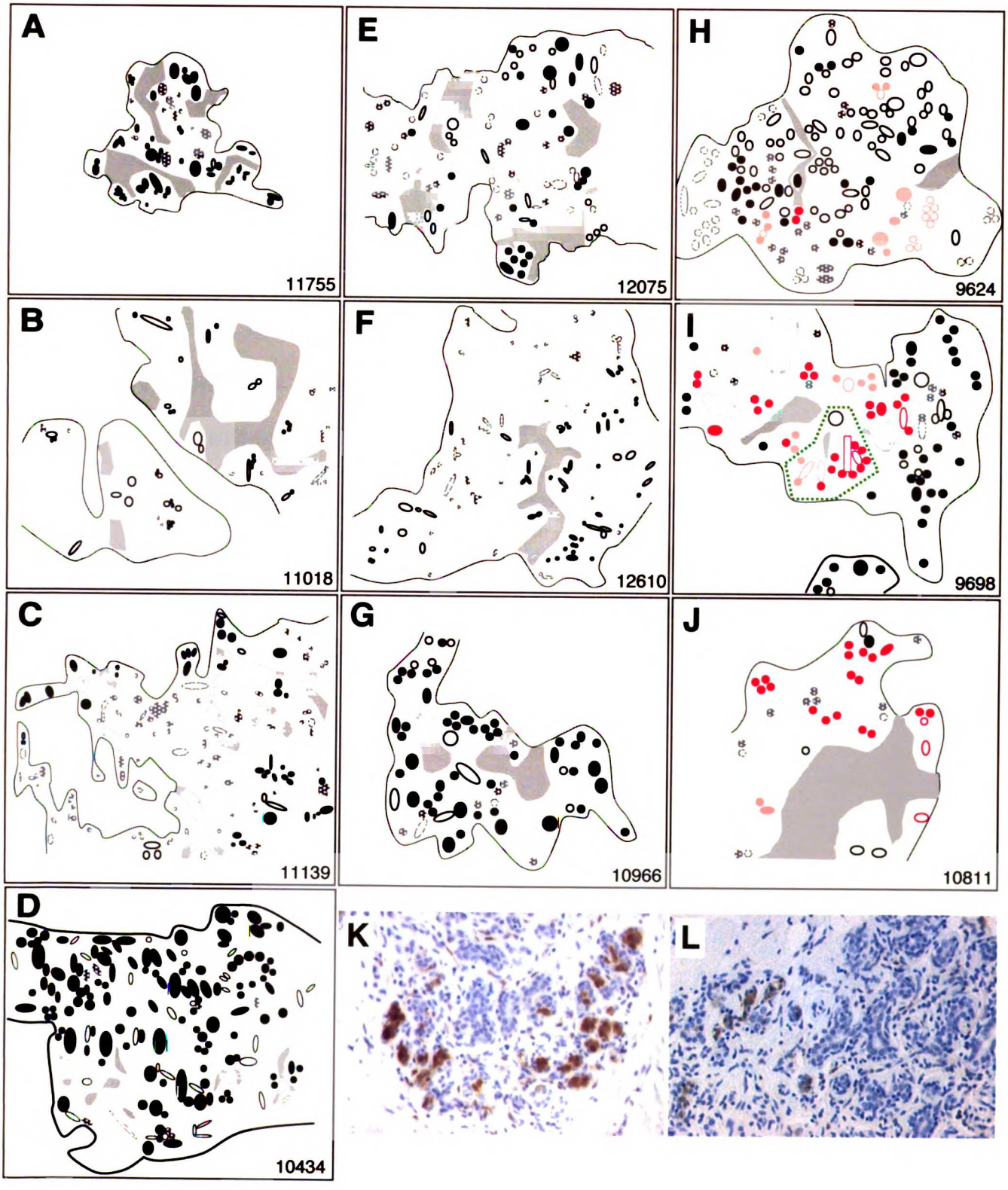
A
11755
E
12075
H
9624
B
11018
F
12610
I
9698
C
11139
G
10966
J
10811
D
10434
K
L

Fig. 4-3. Distribution of epithelial cells with methylated *p16* promoters in histologically normal mammary tissue. *A-J*, we generated a gridded map of epithelial cell clusters by examining the H&E section. We classified epithelial clusters as ductal (*open ovals*) or lobular (*filled ovals*) and then assessed, in the adjacent serial section, the methylation status of each cluster (MSP-ISH). Methylation status of lobular and ductal epithelial cell clusters was scored as negative (*black*, <1% cells methylated), low frequency of positive (*pink*, 1-50% cells methylated), or high frequency of positive (*red*, >50% cells methylated). Epithelial cell clusters that could not be assessed due to processing problems are *stippled gray*. Regions of adipose tissue are represented in *light gray*. *A-G*, examples of tissue without detectable *p16* methylation. *H-J*, examples of tissue containing cells with hypermethylated *p16* promoter sequences. *A-J*, *lower right of panels*, patient identification numbers. *K*, patient 10966, and *L*, patient 10811, representative immunohistochemical detection of p16 protein expression.

CHAPTER 5

Conclusions and Future Directions

Charles R. Holst

ABSTRACT

In concise summary, the following conclusions can be drawn from this dissertation research. First, p16 is necessary for the first population growth plateau of human mammary epithelial cells (HMEC) propagated *in vitro*. Second, variant HMEC possess specific cell cycle checkpoint defects. p16 functions as an arbiter of cell cycle progression under particular conditions, and in particular cell types. In particular, p16 is necessary for preventing S-phase entry upon microtubule disruption in human mammary epithelial cells, but not isogenic fibroblasts. p16 appears to be dispensable for long-term cell cycle arrest in response to DNA damage induced by gamma radiation. Third, the vHMEC subpopulation is not generated at the first plateau, and likely is present very early in the culture and is subsequently selected at the first plateau. Fourth, in support of the previous statement, mammary epithelial cells with *p16* promoter hypermethylation exist prior to explant, in histologically normal breast tissue from individuals without apparent breast disease, the first evidence of vHMEC existing *in vivo*. I will now proceed to elaborate on these points, draw connections to other recent experimental developments in our lab and others, and speculate on the implications of this work.

Dissecting p16-Dependent Phenomena in Human Mammary Cells

By using newly developed RNA interference technology, which allows pseudo-genetic analysis of diploid cell populations, we showed that p16 is necessary for the first growth plateau of HMEC *in vitro*. The careful study by Romanov *et al.* showed that this first plateau of HMEC is phenotypically similar to replicative senescence (Romanov et al., 2001; Tlsty et al., 2001). The first plateau differs from replicative senescence in two fundamental regards. First, with high frequency ($f \approx 10^{-4}$-10^{-6}), variant cells are capable of proliferating beyond the first plateau, an event that has never been observed in fibroblast cultures ($f < 10^{-9}$). Second, the plateau does not apparently depend on the telomere maintenance of the cells, in that expression of the enzymatic subunit of telomerase does not prevent the growth plateau (Kiyono et al., 1998; Stampfer et al., 2001). If the lack of telomere maintenance is unimportant for the initiation of the first plateau, then what does initiate it? Stampfer and Yaswen suggest that oxidative damage may be in some way involved in causing the first plateau. They show that culturing HMEC at an oxygen tension below ambient oxygen levels results in delayed initiation of the first plateau (Yaswen and Stampfer, 2002).

Shay and coworkers argue that "inadequate culture conditions" are responsible for the first plateau of HMEC (Ramirez et al., 2001). They show that the first plateau can be prevented by culturing HMEC in "adequate culture conditions," which involves feeder layers of mitomycin-C killed 3T3 mouse fibroblasts, in medium containing fetal calf serum. However, Shay and coworkers compare the growth curves of HMEC grown in serum-free medium on plastic with HMEC grown in serum-containing medium on feeder layers. As both variables (the presence of serum or feeder layers) may contribute to

HMEC proliferation, it remains difficult to draw any satisfactory conclusion from this experiment. Indeed, Stampfer showed that the proliferation of vHMEC was inhibited by low concentrations of serum (Hammond et al., 1984). These experiments do raise questions about the *in vivo* relevance of the first growth plateau of HMEC. Our attempts to draw parallels between observations in cultured HMEC and cells in histologically normal mammary tissue *in vivo* argue for the relevance of the *in vitro* model system.

A striking and salient phenotype of the vHMEC subpopulation is the abundant chromosomal instability revealed in the cell population approaching agonescence. We hypothesized that deficiencies in cell cycle checkpoint control of vHMEC may be permissive for the full manifestation of this chromosomal instability phenotype. To this end, we showed that vHMEC exhibit specific cell cycle checkpoint defects when compared to isogenic HMEC and HMF populations; namely, (i) vHMEC initiate, but fail to maintain, a DNA damage checkpoint in response to gamma radiation, and (ii) vHMEC populations are unable to arrest in 2N in response to microtubule disruption. These checkpoint defects were surprising in three regards. First, they were surprising in their specificity; vHMEC were competent in certain aspects of checkpoint control (*i.e.*, the 4N arrest after microtubule disruption), but deficient in others. Second, the results were surprising in their differential p16-dependence in HMEC. The microtubule integrity checkpoint was p16-dependent in HMEC, whereas the maintenance of the DNA damage checkpoint was seemingly p16-independent in HMEC. These results make the important suggestion that vHMEC possess additional molecular differences in addition to p16 loss. In other words, these data argue that not all of the vHMEC phenotypes are simply due to their p16 deficiency, and raises the question as to what the additional change(s) is/are.

Third, the results were surprising in that the checkpoints examined in isogenic mammary fibroblasts were both seemingly p16-independent. The *p16* gene therefore functions differently in different cell types. This last observation, along with the cell type specificity of p53 regulation, suggests strongly a path along which to pursue long sought-after epithelial-mesenchymal differences in tumor suppressor function.

Another exciting recent and novel insight into *p16* gene function was provided in experiments done in conjunction with Dr. Kimberley McDermott in our lab (McDermott et al., 2003). She recently discovered that a substantial fraction of vHMEC were unable to prevent centrosome re-duplication when DNA sythesis was experimentally stalled by hydroxyurea (HU) exposure. In contrast, HMEC populations arrested both the DNA replication and centrosome duplication cycles coordinately. Using RNA interference approaches, we subsequently showed that p16 is necessary to prevent centrosome re-duplication upon HU treatment. In other words, p16 acts, in some way, to couple the DNA and centrosome cycles. It will be interesting to test if the role of p16 in coordinating the cell cycle and the centrosome cycle is cell type-specific as well.

Toward an Understanding of the Origin of vHMEC: *In Vitro* and *In Vivo* Observations

The fluctuation analysis described in Chapter 4 indicated that vHMEC are not generated at the time of the first plateau growth arrest. Rather, the variant cells (or precursors thereof) exist prior to the first plateau and have a proliferative advantage, and hence are selected at that time. These results could be explained by a variety of different models, two of which will be proposed here. The first possible model is that vHMEC simply exist *in vivo*, possess all the relevant phenotypic properties, have no proliferative advantage until the time of the first plateau, and exist as a latent and/or lurking subpopulation until selection. The presence of intermediate colony numbers in the fluctuation analysis argues against this simple first model. The second model is that vHMEC exist *in vivo*, but can also be generated *de novo* at some rate during the first exponential phase of proliferation *in vitro*. In this model, HMEC would essentially be "converted" to vHMEC by some single-order process (*i.e.*, *de novo* methylation of the *p16* promoter). This model would explain the intermediate colony numbers in the fluctuation analysis, by postulating that the "jackpot" subpopulations represent vHMEC from *in vivo* and the subpopulations with intermediate colony numbers may represent more recent vHMEC "converts" that arose during the exponential phase of proliferation (but before the growth plateau). It is important to note that the bulk of the results presented in Chapter 2 argue that p16 down-regulation may be the relevant and sufficient change which confers the vHMEC phenotype. vHMEC are an operationally defined population of cells: vHMEC are mammary epithelial cells that possess the ability to proliferate beyond the first growth plateau, under standard, defined culture conditions.

p16 down-regulation in HMEC was sufficient, in three out of four experiments, to prevent the first plateau, and, hence, by the operation definition, sufficient for vHMEC formation.

As more characterization of vHMEC occurs in our laboratory, however, our definition of "vHMEC" has evolved, in that more phenotypes appear to be particular to this special subpopulation of cells (loss of specific cell cycle checkpoint controls, uncoupling of the cell cycle and centrosome cycle, *etc.*) p16 loss in HMEC has not been sufficient to phenocopy all of these additional vHMEC phenotypes, as was evident in the DNA damage checkpoint response phenotype. Indeed, in one out of four RNAi experiments, p16 down-regulation was not sufficient to prevent the first plateau. What, then, is a vHMEC? It appears to be more than simply a p16-null HMEC. Additional, as-yet-undiscovered deficiencies in vHMEC may eventually help to explain all of the phenotypes attributed to this intriguing subpopulation of cells.

Expression profiling of vHMEC populations has revealed a plethora of different gene expression changes between HMEC and vHMEC populations (Y. Crawford, J. Zhang, T.Tlsty, personal communication). Of particular interest were changes in the expression of the COX-2 gene. Specifically in the mammary epithelial cell system, COX-2 was highly expressed in late-passage and agonescent vHMEC, but not in HMEC (Crawford et al., 2003). COX-2 encodes an inducible cyclo-oxygenase enzyme, whose activity results in the production of specific prostaglandins with potent and varied signaling capabilities, including anti-apoptotic, pro-angiogenic, and pro-invasive activities in different systems, including vHMEC (Crawford et al., 2003; Gately, 2000; Tsujii and DuBois, 1995). High COX-2 expression has been demonstrated in invasive

carcinomas of many different types, including breast (Soslow et al., 2000; Tucker et al., 1999). The role of COX-2 in promoting tumorigenesis is being tested in several experimental settings. In particular, overexpression of COX-2 under the control of a mammary gland-specific promoter is sufficient to confer tumor formation in that tissue (Liu et al., 2001). Further experiments have shown that COX-2 activity is necessary for tumor progression in an experimental model of colon cancer (Oshima et al., 1996). COX-2 thus possesses *bona fide* tumor-promoting activity. When examining the prevalence of *p16* promoter methylation *in vivo*, Crawford *et al.* found that intense COX-2 staining was only found in reduction mammoplasty specimens that also contained p16 promoter hypermethylation (Crawford et al., 2003). Furthermore, the foci of p16 promoter hypermethylation in large part overlapped the COX-2 expression. This *in vivo* co-localization of COX-2 overexpression and *p16* promoter hypermethylation, two functionally relevant markers of vHMEC *in vitro*, strongly supports the contention that cells comparable to the vHMEC (as defined *in vitro*) also exist *in vivo*. This is a hypothesis that is currently and enthusiastically being tested in our laboratory.

The significance of observing cells with properties of vHMEC *in vivo*, in a substantial fraction of women with no *known* increased risk for breast cancer, is not yet known, and may in fact be profound. Large-scale studies of disease-free women with and without indication of *p16* hypermethylation and/or COX-2 overexpression will be necessary to determine the long-term significance of the observations. As mentioned above, both of these molecular alterations have been associated with cancer, and one prediction would be that "normal" women with either or both molecular alteration(s) might be at an increased risk for developing cancer in their lifetimes. In this case, these

molecular alterations may be assayed as early biomarkers of cancer risk (Laird, 2003). Interestingly, highly sensitive assays for *p16* hypermethylation in patients at high risk for lung cancer are already being considered (Belinsky et al., 1998). Alternatively, if neither molecular change is associated with increased risk for breast cancer, then the implications are none the less profound, in that they call into question the assumption by many researchers that the simple observation of certain markers in tumors implies functional relevance. The question arises: where does physiology end and pathology begin? Only a thorough study of the "normal" situation, with long-term clinical follow-up, will allow satisfactory elucidation of this question.

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APPENDIX

Normal Human Mammary Epithelial Cells Spontaneously Escape "Senescence" and Acquire Genomic Changes

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CREDITS

The vast majority of the work in this appendix chapter was done by Serguei Romanov and Krystyna Kozakiewicz. I assisted in the data analysis and presentation, the writing and assembling of the manuscript, as well as providing intellectual input.

ABSTRACT

Senescence and genomic integrity are believed to be important barriers to the development of malignant lesions[1]. Human fibroblasts undergo a limited number of cell divisions prior to entry into an irreversible arrest, senescence[2]. Human mammary epithelial cells (HMECs) do not conform to this paradigm of senescence. In contrast to fibroblasts, HMECs exhibit an initial phase of active growth, enter a transient growth plateau (termed selection or M0[3-5]) from which proliferative cells emerge, undergo additional population doublings (~20-70) before entering in a second growth plateau, previously termed senescence or M1[4-6]. We find that the first growth plateau exhibits characteristics of senescence but is not an insurmountable barrier to further growth. HMECs emerge from senescence, exhibit eroding telomeric sequences and ultimately enter telomere-based crisis to generate the types of chromosomal abnormalities seen in the earliest lesions of breast cancer. Thus, growth past senescent barriers may be a pivotal event in the earliest steps of carcinogenesis providing multiple genetic changes predicating oncogenic evolution. These differences between epithelial cells and fibroblasts provide new insights into the mechanistic basis of neoplastic transformation.

Results and Discussion

To analyze these cell-specific differences, we characterized the *in vitro* proliferation barriers in isogenic HMECs and human mammary fibroblasts (HMFs) from healthy individuals [3,7,8]. Similar to previous studies in human skin fibroblasts[1] and HMECs [3-5,9], both the epithelial and fibroblast cell populations underwent a limited number of population doublings prior to entering a plateau (Fig. A-1, HMF phase b, HMEC phase b) (variously termed the Hayflick limit[1], irreversible replicative senescence, and mortality stage 1 (M1)[2]). The cells enlarged in size, flattened in shape, became vacuolated (Fig. A-1), and expressed senescence-associated β-galactosidase [10] (SA-β-gal, data not shown). Low incorporation of bromodeoxyuridine (BrdU) and minimal presence of MCM2 protein indicated a low proliferative index. Additionally, Annexin-V staining indicated a low death index (data not shown). Further characterization demonstrated that human foreskin fibroblasts, pre-selection HMECs and HMFs each: 1) maintained genomic integrity (Fig. A-2; ref. 8); 2) maintained intact cell cycle checkpoint control (data not shown); 3) exhibited a 2N to 4N DNA content ratio of ≥ 4 at phase b (Table A-1); and 4) had a mean TRF length that was similar at senescence (Fig. A-3). By the morphological, behavioral and molecular criteria described above, HMFs and HMECs senesce in a manner similar to human skin fibroblasts such that "M0" of HMECs corresponds to "M1" of fibroblasts.

Strikingly, the ability of HMECs and HMFs to spontaneously overcome senescence differ by several orders of magnitude. In skin fibroblasts, senescence can last for years (at least 3 years, TDT unpublished data), cells remain viable if fed routinely [12], and the frequency of spontaneous emergence is $<10^{-9}$ (data not shown; ref. 13).

Similarly, HMFs failed to produce proliferating cells from senescent populations even after 5 months in continuous culture in either serum-containing or serum-free media (<6 x 10^{-7}, data not shown). In contrast to fibroblasts and consistent with previous reports [3,5], HMECs maintained at the first plateau in serum-free media sporadically emerge at a high frequency and generate clusters of small, refractile cells (Fig. A-1, HMEC c; 1.43 ± 0.04 x 10^{-4} (donor 48, mean ± SD, n=4) and 1 ± 1 x 10^{-5} (donor 184, n=4)). Pre- and post-selection HMECs demonstrated typical heterogeneous expression of cytokeratins when examined by immuno-cytochemistry (data not shown; ref.14). As reported previously, HMECs that emerge from the first plateau (phase b in Fig. A-1) lose expression of the p16 protein (supplementary information; ref. 5,9,15).

After a second period of exponential growth (Fig. A-1, HMEC phase c), HMECs entered a second growth plateau (Fig. A-1, HMEC phase d). Unexpectedly, this plateau was critically different from the senescent arrested state in that the cells displayed many hallmarks of cell crisis. HMECs at this stage were heterogeneous in size and morphology (Fig. A-1, HMEC phase d) and demonstrated SA-β-gal staining (data not shown). Furthermore, they continued to incorporate BrdU (16.3 ± 1.1%, 4 hr. pulse, n=2) and retained high levels of MCM2 protein (> 50% of nuclei strongly staining for MCM2, data not shown). Upon FACS analysis, the 2N to 4N DNA ratio was approximately 1 (data not shown), typical of a population of cells in crisis [2]. This high proliferative index was counterbalanced by an increase in cell death. A significant fraction (~20%) of epithelial cells at the second plateau stained with Annexin-V, an indicator of cell death. In contrast, <1% of isogenic senescent HMFs or HMECs at the first plateau were Annexin-V-positive. While DNA fragmentation characteristic of apoptosis was not detectable by

TUNEL assay, we did observe significant fragmentation of nuclei (micronucleation) in these cells as documented by DNA staining of interphase nuclei (data not shown). Thus, HMECs at the second plateau were fundamentally different than HMECs at the first plateau or fibroblast cells at senescence. They exhibited many of the characteristics of viral oncoprotein-induced crisis, with the important exception that no immortalized variants have been detected.

Cytogenetic analysis of post-selection HMECs at selected passages demonstrated that gross chromosomal abnormalities appeared in virtually every metaphase spread as the cells approached the second growth plateau (Fig. A-2). In all cases, including several HMEC populations obtained commercially, the abnormalities accumulated rapidly, beginning 10-20 population doublings before the final passage of cells (Fig. A-2 a) and coincided with slowing of the proliferation rates. In these cells, both the percent of abnormal metaphases and the number of abnormalities per metaphase increased. The abnormalities included multiple translocations, deletions, other rearrangements, telomeric associations, polyploidy and aneuploidy (Fig. A-2 b, c, d). Substantial polyploidy (~25%) was detected by flow analysis at final passages of post-senescent HMECs (data not shown). Microscopy revealed anaphase bridges and failed cytokineses (data not shown). The accumulation of chromosomal abnormalities was independent of donor age (range = 16 - 50 y) and total proliferative potential of epithelial populations (range = 30 - 60 PD).

The timing and spectrum of chromosomal abnormalities, especially the numerous telomeric associations, suggested that late-passage HMECs had entered telomere-based crisis [17,18]. Therefore, various aspects of telomere metabolism were assessed in serial

subcultures of HMECs and HMFs. Both cell populations lacked telomerase activity (data not shown; ref. 16) and exhibited a similar rate of telomere erosion (approximately 30 bp per population doubling; data not shown). As mentioned above, mean TRF lengths in isogenic HMECs at the first growth plateau and HMF at senescence were equivalent and similar to that in the earliest available passage of post-selection cells. Further proliferation of the post-selection HMECs was accompanied by continued shortening of the telomeres (Fig. A-3a) down to a broad range of mean TRF lengths (mean ~3.5 kbp) at the second plateau. Length distribution of telomeres at each plateau was also assessed by quantitative analysis of fluorescence *in situ* hybridization of telomeric repeats [17] (Q-FISH). Consistent with the above determination of mean TRF, mean fluorescence intensity at individual telomeres was diminished significantly (>55%) in HMECs at the second plateau compared to those at the first plateau (Fig. A-3 c,d). In addition, the average number of telomeres with no signal per metaphase spread was ~4 and ~2 in senescent fibroblasts and epithelial cells at the first plateau, respectively, and increased to ~18 in epithelial cells at the second plateau. Thus, the HMECs that emerged from senescence ultimately entered telomere-based crisis.

There are striking parallels between late-generation telomerase-deficient p53 mutant mice and late-passage post-selection HMECs. In wild-type mice, the activation of p53 mediates response to telomere dysfunction [19], leading to p21 induction and cell cycle arrest[20, 21]. Cells from the p53-mutant mice, like the post-selection HMECs, lack telomerase activity, and lack the p53-dependent arrest induced by critically shortened or dysfunctional telomeres. In both cell populations, these conditions generate similar types

of chromosomal abnormalities. However, HMECs only attain this state upon silencing p16 and emerging from senescence.

We assessed p53, its modulator $p14^{ARF}$ and a downstream effector, p21, in serial subcultures of HMFs and HMECs (supplementary information). Consistent with previous reports of these proteins in human skin fibroblasts [22,23], as HMFs were grown to senescence they exhibited minimal changes in total p53 protein levels, a modest increase followed by a slight decrease of p21 protein expression and an increase in p16 protein levels. Similar expression was seen in HMECs at the first growth plateau. Myc protein did not change during epithelial cell culture. Contrary to expectations, we observed an increase in total p53 protein levels in HMECs upon their emergence from senescence but not upon induction of senescence (phase b) or onset of telomere-based crisis (phase d). An increase in $p14^{ARF}$ and p21 and a decrease in p16 accompanied this up-regulation of p53. Both p53 and p21 proteins showed nuclear localization in post-selection cells (data not shown). While the majority of post-selection HMEC populations retained a constant level of p53 protein through crisis, only one sample (donor 48) showed further elevation of p53 protein at crisis that was not accompanied by further increases in $p14^{ARF}$ or p21 (supplementary information). These data suggest that, as HMECs emerge from senescence, unidentified signals activate the p53 pathway but fail to explain why these post-selection cells can proliferate in the presence of elevated p53 and p21.

These studies have several important ramifications. First, they challenge traditional views of how and when cells acquire genomic changes in cancer by providing a cell-intrinsic mechanism that, early in the neoplastic process, generates multiple simultaneous genetic changes without obligatory exposure to physical, viral or chemical

mutagenic agents. Second, although post-selection HMECs have commonly been regarded as normal [4,5], the present observations refute this assumption. These cells do not express p16 [5,9,15], they lack proper checkpoint control [7] and they do not maintain genomic integrity (Fig. A-2). Third, these findings redefine a high frequency spontaneous event that occurs in HMECs and demonstrate that "M0", not "M1", is actually senescence. HMECs spontaneously emerge from senescence, whereas isogenic fibroblasts do not. Should these cells arise *in vivo* (a proposal consistent with preliminary observations), they would provide generative material for human carcinogenesis. Therefore, our observations that HMEC proliferation beyond senescence leads to telomeric dysfunction, coupled with published observations that telomeric dysfunction leads to carcinogenesis in mice [20,21], could explain the early steps in carcinogenesis. Finally, these observations identify novel clinical opportunities. They provide potential markers for assessing susceptibility to neoplastic transformation in individuals as well as potential targets for prevention and therapy. Multiple markers (Table A-1; ref. 25) clearly identify the different cellular states and may allow the identification of these cells *in vivo*. Remarkably, the earliest lesions in breast cancer, hyperplasias, demonstrate abnormally controlled proliferation but relatively few chromosomal structural abnormalities [25], a phenotype similar to early-passage post-selection HMECs. The more progressed lesions in breast cancer, DCIS (ductal carcinoma *in situ*), in addition demonstrates the types of chromosomal aberrations observed in late-passage HMECs [26]. We hypothesize that the above-described properties of HMECs *in vitro* are critically relevant to their transformation processes *in vivo*. Agents that minimize HMEC transition past the growth plateaus should decrease the incidence of breast cancer. Given that irreversible

senescence and tight control of genomic stability are believed to be important barriers for the development of cancerous lesions, our observations also suggest a much higher risk of neoplastic transformation originating in mammary epithelial tissue than in mesenchymal tissue, a prediction consistent with extensive epidemiological studies [27].

METHODS

Cells and cell culture. Isogenic sets of human breast epithelial cells and fibroblasts (*i.e.*, from the same gland) were previously generated [3]. Briefly, tissue from reduction mammoplasty was digested to epithelial organoids and the accompanying fibroblasts. Cells from donors 48 and 184 were obtained from a 16 year-old and a 21 year-old woman, respectively, and showed no pathologic epithelial cells. Epithelial cells were grown and subcultured using two different media: MM, which contains 0.5% fetal bovine serum and several growth factors, and MEGM, a serum-free medium containing growth factors and bovine pituitary extract. Cell doubling times were 18-24 hours in either medium. Three additional populations of human breast epithelial cells, all derived from reduction mammoplasty (1001-3, 4144-2 and 4678-2) were purchased from BioWhittaker (USA). Fibroblasts were grown in DMEM with 10% fetal calf serum. Cells were grown at 37°C in 5% CO_2. For routine culture, cells were counted and plated at $2x10^5$ cells per 75-cm^2 flask. Attachment efficiency was determined by counting attached cells 15 h after plating. The number of accumulated populations doublings (PD) per passage was determined using the equation, PD=log(A/(BxC))/log2, where A is the number of harvested cells, B is the number of plated cells, and C the attachment efficiency.

Chromosomal analysis. Metaphase spreads were prepared from cells treated with Colcemid® (KaryoMAX, GibcoBRL, 100 ng/ml for 6 h). We performed standard G-banding karyotypic analysis on at least 50 metaphase spreads for each population.

Metaphase spreads were classified as abnormal if they contained any complement of chromosomes besides 46 XX with normal banding patterns.

Cell cycle analysis. Cells were plated at an initial density of 10^5 cells per 100-cm^2 flask. Cells were metabolically labeled with BrdU (10 μM, 4 h), trypsinized, and fixed with 70% ethanol. Nuclei were isolated and stained with propidium iodide and FITC-conjugated anti-BrdU antibodies (Becton Dickinson, USA), as previously described [7]. Flow cytometry was performed on FACS-Sorter (Becton Dickinson). All analyzed events were gated to remove debris and aggregates.

Cell death assays. TUNEL assay for DNA fragmentation was done using the In Situ Cell Death Detection kit (BMB), according to manufacturer's protocol. Alternatively, living cells were stained with Annexin-V-FLUOR (BMB) and propidium iodide and analyzed by fluorescent microscopy.

Telomere length assay. Genomic DNA (10 μg), isolated from cultured cells, was digested with restriction enzymes Rsa I and Hinf I and then separated in a 0.5% agarose gel. DNA was transferred to Hybond-N^+ membrane (Amersham, UK). Blots were probed with 5' end-labeled oligonucleotide $(TTAGGG)_6$ end exposed to a PhosphoImager plate to detect the telomeric ends. An average telomere length was determined using ImageQuant software (Molecular Dynamics, USA).

Quantitative *in situ* hybridization with telomeric probe (Q-FISH). *In situ* hybridization of telomere-specific peptide nucleic acid probe (Telomere PNA FISH Kit/Cy3, DAKO) to metaphase chromosome was performed according to the manufacturer's protocol using metaphase spreads prepared from cells treated with Colcemid. Images were captured by a CCD camera attached to a Nikon TE300 microscope and analyzed using IPLab Spectrum (Scanalytics Inc.). Background was subtracted and fluorescent signal was integrated in segments corresponding to individual telomeres [17].

Senescence-associated β-galactosidase assay. Senescence-associated β-galactosidase was detected in fixed cells using the protocol described previously [10]. When staining was fully developed, the cells were washed with PBS and incubated with propidium iodide (1 μg/ml in PBS) and with DNase-free RNase A (5 μg/ml). Both phase-contrast and fluorescent microscopy were performed to identify senescent cells and their nuclei.

Western analysis. Total cell extracts were fractionated in gradient (4%-20%) polyacrylamide gels (FMC) and transferred to Hybond-P (Amersham) membrane. Antibodies: mouse monoclonal anti-human $p16^{ink4a}$ (NeoMarkers, AB-1), mouse anti-p53 (Santa Cruz, DO-1), mouse anti-p21(WAF1) (Calbiochem, Ab-1), rabbit anti-$p14^{ARF}$ (NeoMarkers, Ab-1) and mouse anti-a-actin (Sigma); HRP-conjugated goat-anti-mouse antibody (Gibco) and goat anti-rabbit antibody (Calbiochem). Staining was developed using ECL-detection protocol.

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Table A-1. *Summary of Human Cell Characteristics at Different Growth Plateaus*

	Cells				
	Fibroblasts		Mammary Epithelial Cells		
Characteristic	Senescence	Crisis[a]	1st Plateau	2nd Plateau	Crisis[a]
Lack of increase in cell number	+	+	+	+	+
SA-β-gal staining	+	+	+	+	+
BrdU incorporation	-	+	-	+	+
Cell death	-	+	-	+	+
Genomic Instability	-	+	-	+	+
2N/4N ratio	>4	~1	>4	~1	?
Polyploidy	low	high	low	high	?
MCM2 expression	low	high	low	high	?
Population expansion beyond growth plateau	-	+	+	?	+
Existing nomenclature	Hayflick Limit, Senescence, Irreversible replicative senescence	Crisis, Apparent proliferative arrest	Selection, Senescence, Terminal arrest	Senescence, Replicative senescence	Crisis
	M1	M2	M0	M1	M2
Proposed nomenclature	**same**	**same**	**selection**	**Agonescence**	**same**

[a] Properties descriptive of viral oncoprotein-induced crisis

Figure A-1

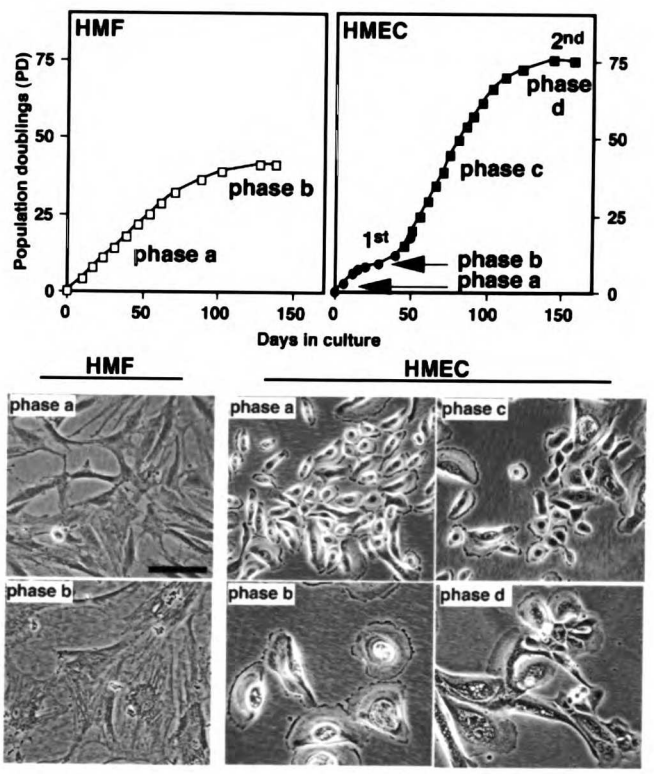
HMF
HMEC
Population doublings (PD)
75
50
25
0
phase a
phase b
1st
2nd
phase c
phase d
0
50
100
150
Days in culture
HMF
HMEC
phase a
phase b
phase a
phase c
phase b
phase d

Fig. A-1. HMF and HMEC growth curves and cell morphologies *in vitro*.

Tissue was dissociated with collagenase and hyaluronidase and plated in parallel cultures, one in medium (DMEM) that supported the growth of fibroblasts and the other in medium (MEGM) that supported the growth of epithelial cells. The growth curve and microscopic morphology of both mammary fibroblast and epithelial cells from donor 48 during the first phase of logarithmic growth (phase a) and the first growth plateau (phase b) is shown for each population. The second epithelial phase of proliferation (phase c) and the second epithelial growth plateau (phase d) are also shown. Scale bar, 100 μm.

Figure A-2

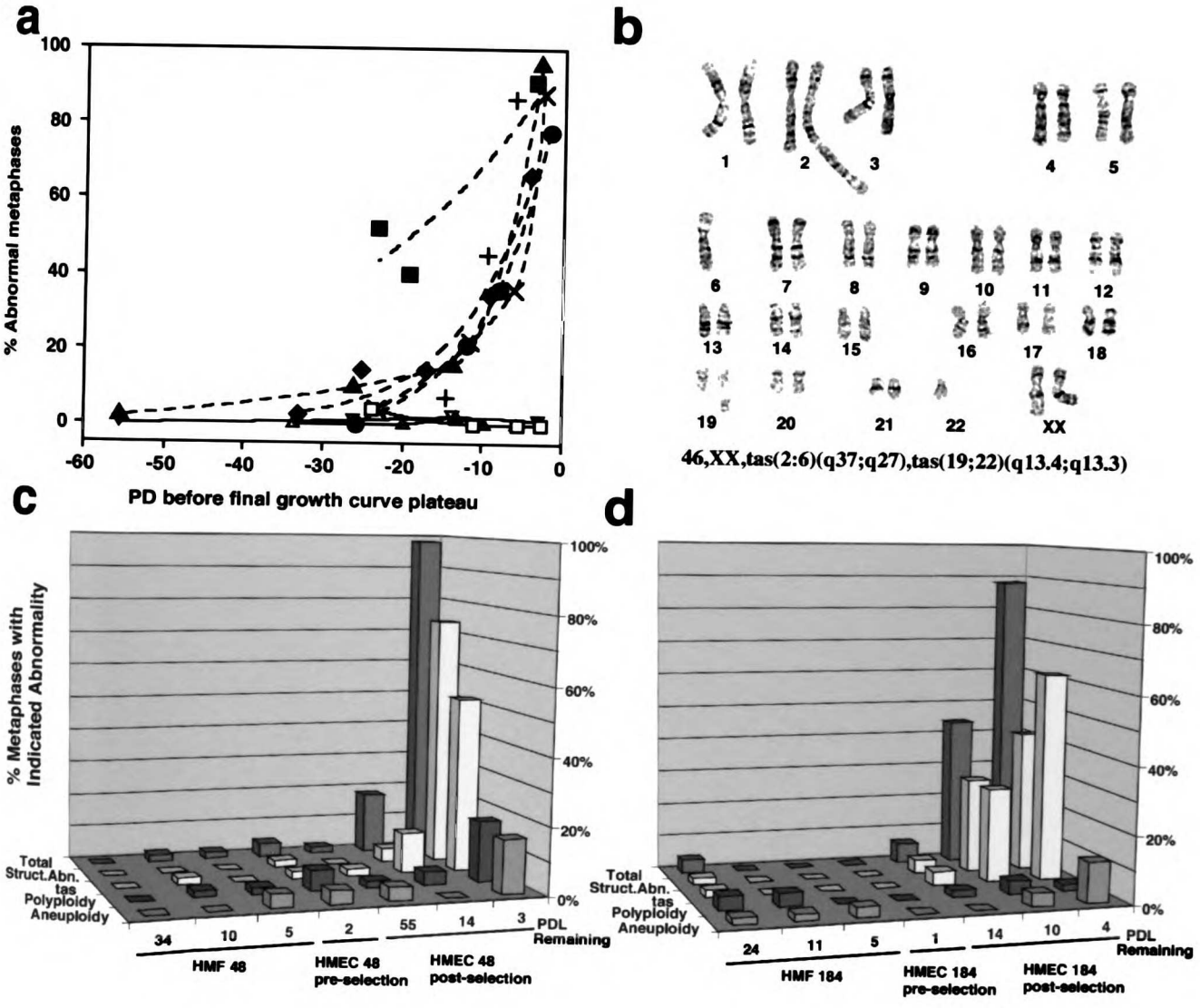
a
% Abnormal metaphases
PD before final growth curve plateau
b
46,XX,tas(2:6)(q37;q27),tas(19;22)(q13.4;q13.3)
c
% Metaphases with Indicated Abnormality
Total Struct.Abn. tas
Polyploidy
Aneuploidy
HMF 48
HMEC 48 pre-selection
HMEC 48 post-selection
PDL Remaining
d
HMF 184
HMEC 184 pre-selection
HMEC 184 post-selection
PDL Remaining

Fig. A-2. Spontaneous genomic instability in human mammary epithelial cells. (**a**) Kinetics of accumulation of karyotypic abnormalities. The percentages of metaphase spreads with structural chromosomal abnormalities were determined as a function of the number of population doublings before final growth plateaus (0 PD). HMF populations 48 (Δ) and 184 (❑). HMEC populations 48 (▲), 184 spiral K (■), 184 birdie (+), 4678-2 (X), 1001-3 (●) and 4144-1 (◆). (**b**) Representative abnormal karyotype from HMEC 48. (**c,d**) Types of chromosomal abnormalities observed in HMEC 48 and 184, respectively. Definitions: Total Abnormalities (all structural abnormalities and telomeric associations, not including numerical abnormalities), Struct. Abn. (deletions, duplications, rings, marker chromosomes, chromatid exchanges, and translocations), tas (telomeric associations), polyploidy (multiples of a diploid chromosome complement), aneuploidy (additions or deletions of whole chromosomes).

Figure A-3

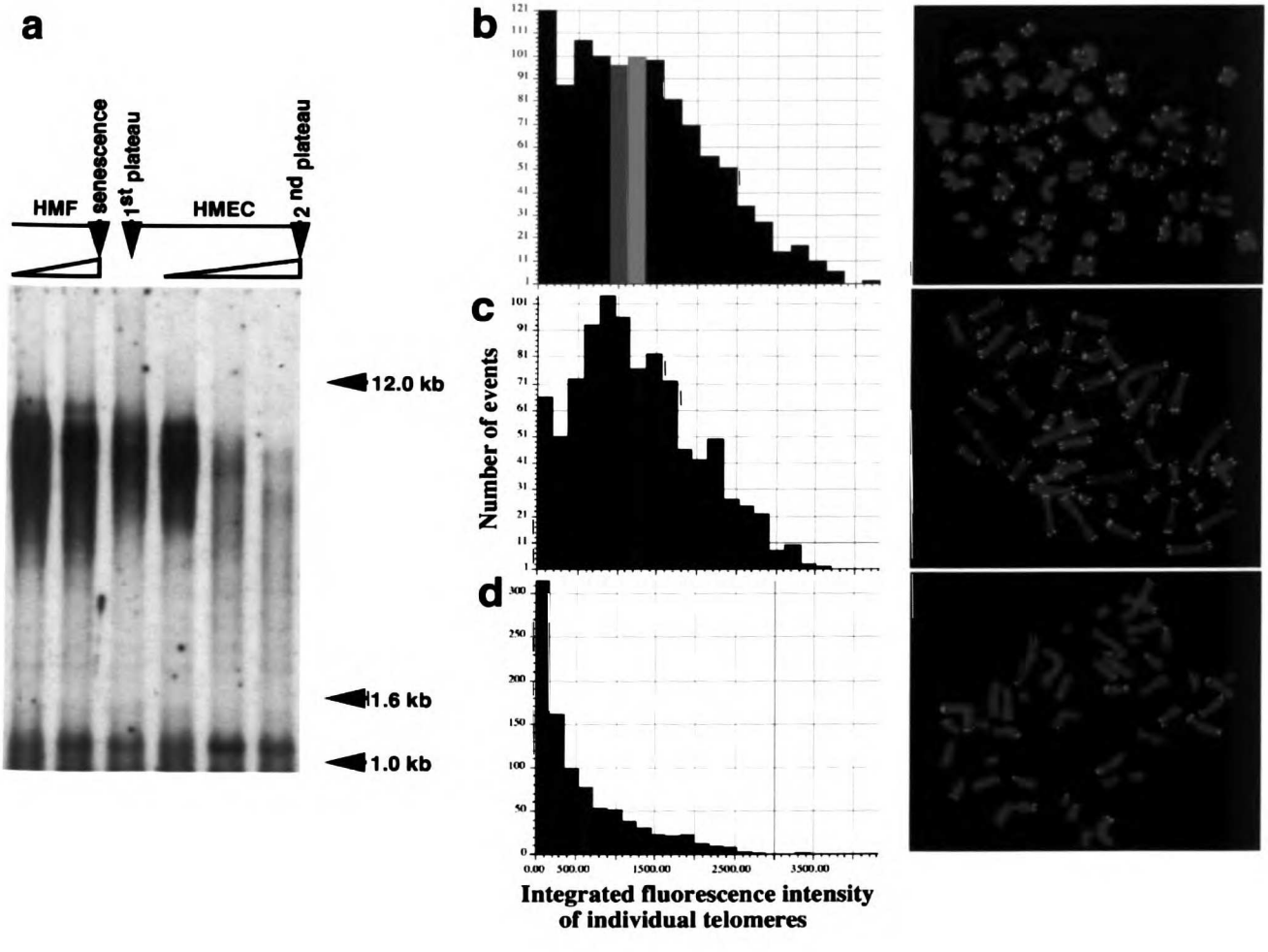
a
HMF
senescence
1st plateau
HMEC
2nd plateau
12.0 kb
1.6 kb
1.0 kb
b
c
d
Number of events
Integrated fluorescence intensity
of individual telomeres

Fig. A-3. Post-selection HMEC continue to shorten telomeres beyond the length detected in senescent HMF and HMEC at the first growth plateau. (**a**) TRF analysis of HMF 48 (PD10), senescent HMF 48 (PD 42), pre-selection HMEC 48 at first growth plateau (PD 12), and post-selection HMEC 48 at PD20, PD65 and at second growth plateau (PD 75). (**b-d**) Quantitative FISH (Q-FISH) analysis of telomeric repeats in senescent HMF 48 (b), HMEC 48 cells at first growth plateau (c) and HMEC 48 cells at second growth plateau (d). Shown are representative images of in situ hybridization of Cy3-$(C_3TA_2)_3$ PNA probe (red) to metaphase chromosomes (blue) and histograms of distribution of integrated fluorescence intensities of >1000 individual telomeres. Red and green bars indicate the position of mean and median values, respectively, for each data set.

Figure A-4

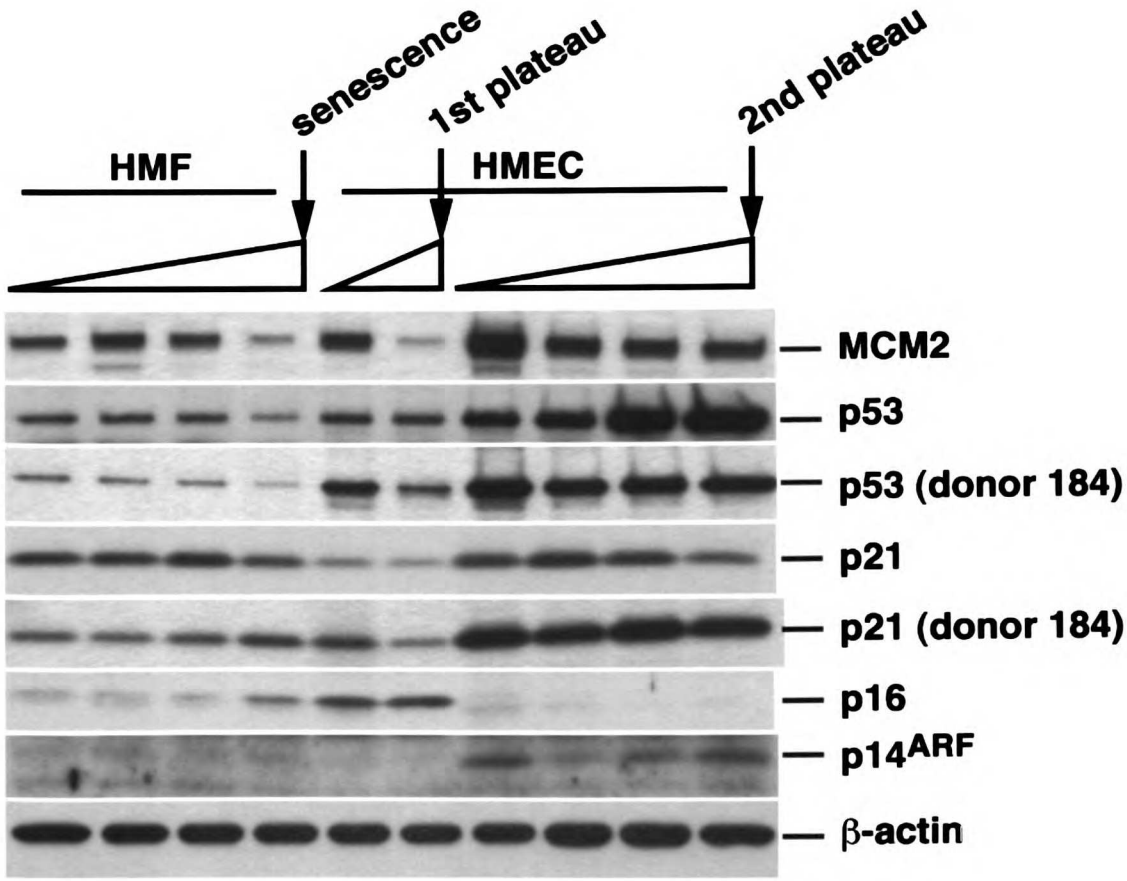
senescence
1st plateau
2nd plateau
HMF
HMEC
MCM2
p53
p53 (donor 184)
p21
p21 (donor 184)
p16
p14ARF
β-actin

Fig. A-4. Western analysis of protein expression in human mammary cells. Changes in p53, p21, $p16^{INK4A}$ and $p14^{ARF}$ protein expression were assessed by Western analysis at various stages of growth of isogenic HMECs and HMFs. HMF 48 were assessed at PD 3, 20, 33 and 35 (senescence). Pre-selection HMEC 48 cells were analyzed at PD 10 (before the first growth plateau) and 12 (at the first growth plateau). Post-selection HMEC 48 cells were analyzed at PD 20 (soon after emergence from the first plateau), 60, 70 and 75 (second growth plateau). Also shown is the status of p53 and p21 proteins in HMF 184 cells at PD10, 17, 24 and 25 (senescence), in pre-selection HMEC 184 cells at PD9, 12 (first growth plateau) and in post-selection HMEC 184 birdie cells at PD30, 35, 37 and 38 (second growth plateau). Staining for β-actin was performed to verify even loading. Triangles indicate increasing time in culture.