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IDENTIFYING EARLY STEPS OF BREAST CARCINOGENESIS

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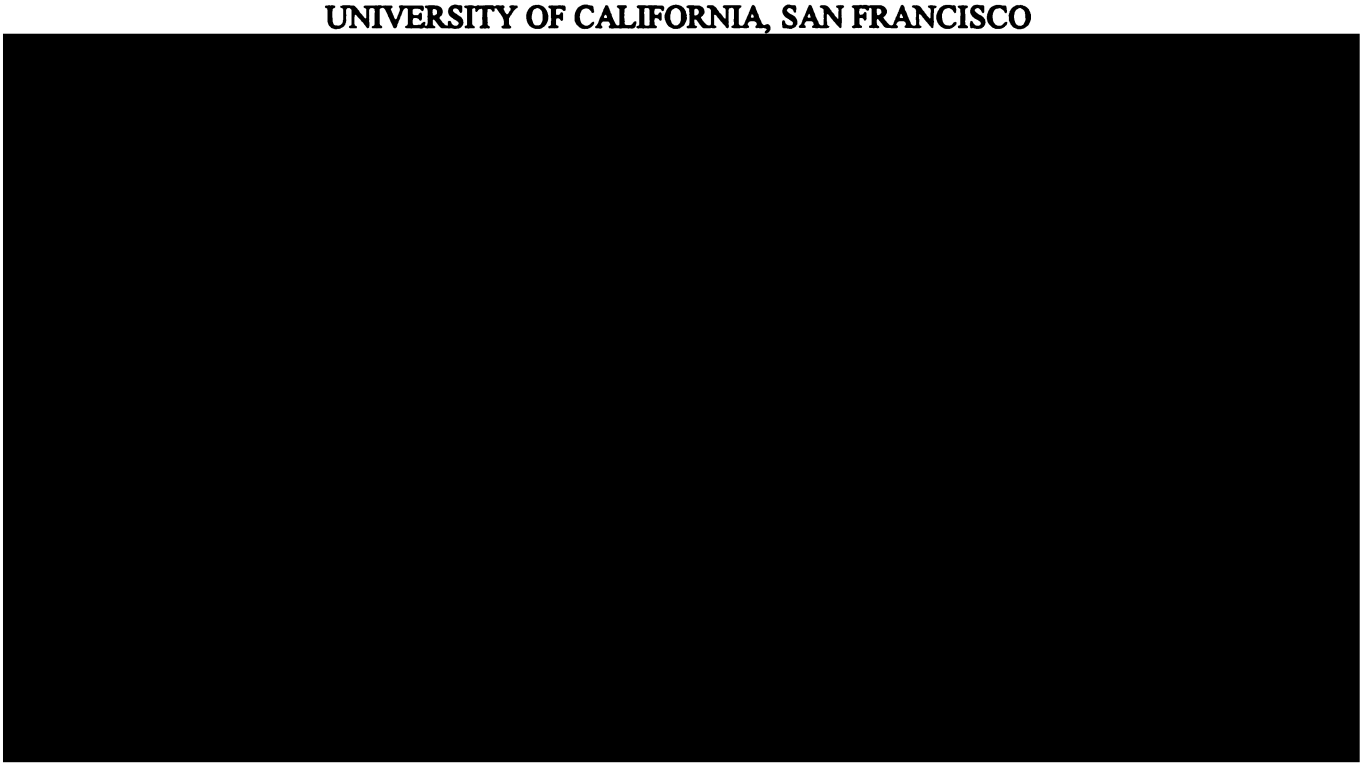

To My Family

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IDENTIFYING EARLY STEPS OF BREAST CARCINOGENESIS

Yongping Crawford

Abstract

Breast cancer is one of the most common causes of cancer death in western women and is also a major health concern for women worldwide. It is characterized by widespread genetic and epigenetic alterations that activate oncogenes and/or inactivate tumor suppressors. Studies from murine models coupled with clinical observations have provided invaluable insights into breast cancer initiation and progression. However, the mechanisms that initiate the malignant transformation of human mammary epithelial cells remain to be fully elucidated.

Breast tissue from healthy women with no indication or predisposition to breast cancer contains a subpopulation of variant mammary epithelial cells (vHMEC) exhibiting $p16^{INK4a}$ promoter hypermethylation both *in vivo* and *in vitro*. When continuously cultured, vHMEC acquire telomeric dysfunction and produce the types of chromosomal abnormalities seen in pre-malignant and malignant lesions of the breast. We find that late passage vHMEC, or vHMEC exposed to DNA damaging agents, express elevated prostaglandin cyclo-oxygenase 2 (COX-2). This over-expression of COX-2 protein in vHMEC specifically contributes to phenotypes usually associated with malignant cells including increased prostaglandin E_2 synthesis, angiogenic potential, invasive ability, and decreased apoptosis. Moreover, COX-2 over-expression coincides with focal areas of $p16^{INK4A}$ promoter hypermethylation *in vivo* creating ideal candidates as precursors to

breast cancer. Taken together, these data indicate that COX-2 over-expression and *p16^{INK4a}* promoter hypermethylation, often seen in the late stages of breast cancer, occur early during the breast carcinogenic process and may in fact be the driving forces for breast cancer initiation.

To prevent vHMEC from further proliferation and progression, we exploited the difference between HMEC and vHMEC in COX-2 expression as well as p16 expression and the downstream E2F activity. We find that vHMEC with pre-malignant phenotypes can be preferentially eliminated upon exposures to either COX-2 specific inhibitor or engineered adenovirus, Onyx 411, that targets cells with deregulated E2F activity.

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Chapter 1

Introduction

Yongping G. Crawford

Introduction

Breast Cancer Statistics and Risk Factors

Breast cancer is the most frequently diagnosed cancer in women (~30.7 %) (2002 cancer data, http://www.naaccr.org) and is also one of the most common causes of cancer death in women (SEER Cancer Statistics Review 1975-2001, http://www.nci.nih.gov). Approximately, one in eight women (~13.3%) living in the United States will develop breast cancer in her lifetime (the 2004 National Cancer Institute report http://cis.nci.nih.gov/fact/5_6.htm). Given the prevalence and severity of the disease, identifying the risk factors that affect breast cancer rate and understanding the molecular mechanism of breast cancer initiation, progression, maintenance, and invasion will be necessary to advance our knowledge of the disease and facilitate breast cancer prevention, diagnosis, monitoring, and treatment.

Thus far, epidemiological studies have identified many risk factors that affect breast cancer rate. Among them the onset of menses, parity, pregnancy, and menopause have been documented to be important determinants (Page et al., 2000). All of these risk factors are related to hormone status suggesting they may be the major underlying factors for breast cancer risk. Similarly, many studies also indicate that hormone replacement therapy (HRT) usage also increases breast cancer risk (Chlebowski et al., 2003; Colditz et al., 1995), though this result still remains controversial (Warren and Halpert, 2004). Additional factors such as antibiotic usage (Velicer et al., 2004), obesity, and radiation exposure can also influence breast cancer rate (http://www.nci.nih.gov).

Even though environmental factors clearly influence breast cancer rate, breast cancer can still be characterized as a genetic disease. Accumulation of genetic and epigenetic alterations, which translates into aberrant cellular activities, is the putative cause for breast cancer. Detailed discussions regarding the genetic and epigenetic alterations that activate oncogenes and inactivate tumor suppressors in both human and murine mammary gland malignancies are the central topics of this introduction.

Genomic Instability Contributes to the Hallmarks of Breast Cancer

One common characteristic for all breast cancers, or any other malignancy, is the presence of wide spread genetic and epigenetic abnormalities (Chin et al., 2004; Szyf et al., 2004; Teixeira et al., 2002). In fact, substantial genomic abnormalities are seen not only in invasive breast cancers but also in premalignant breast lesions such as ductal hyperplasia and carcinoma *in situ* (DCIS) (Chin et al., 2004). Similarly, Romanov *et al.* reported that a variant subpopulation of human mammary epithelial cells (vHMEC) isolated from women with no indication or predisposition to breast cancer exhibits both genetic and epigenetic alterations (Romanov et al., 2001; Tlsty et al., 2001). The existence of genomic abnormalities in both premalignant and malignant lesions of the breast suggests that genomic instability may be one of the early driving forces for malignant transformation. This observation is consistent with the Nowell hypothesis that genomic instability provides heterogeneity required for the evolution of variant sub-lines with increasing growth advantages (Nowell, 1976).

Genomic instability plays a crucial role in initiating and promoting the expression of malignant phenotypes described as the "Hallmarks of Cancer" (Hanahan and

Weinberg, 2000). Underlying genomic instability may be a direct driving force for breast cancer cells as they acquire self-sufficiency in proliferative growth signals, insensitivity to growth inhibitory signals, ability to evade apoptosis, limitless replication potential, sustained angiogenesis, and ability to invade and metastasize. For instance, activation of Ras or Her-2/neu, due to either mutation or gene amplification, stimulates cellular proliferation in the absence of mitogenic signals. Likewise, mutation or gene amplification of cyclin-dependent kinase inhibitors or cyclin-dependent kinases renders cells resistant to growth inhibitory signals. In some cases, up-regulation of a single gene such as cyclo-oxygenase 2 (COX-2) can simultaneously confer the majority of the phenotypes described as the "Hallmarks of Cancer". The molecular function and significance of COX-2 in promoting premalignant phenotypes will be discussed in the Chapter 2.

Even though histologically breast cancer is largely an epithelial disease, genomic abnormalities seen in breast cancers are not limited to the cancerous epithelial cells (Moinfar et al., 2000; Wernert et al., 2001). Moinfar *et al.* reported that the normal appearing stromal components surrounding either DCIS or invasive ductal carcinoma (IDC) exhibited substantial amounts of loss of heterozygosity (LOH) whereas stromal components from the control breast without any disease did not (Moinfar et al., 2000). Moreover, some of the same LOH seen in the pre-malignant or malignant epithelial cells were also present in the normal appearing stroma surrounding the lesion. The author suggested that stromal alterations might precede the genetic alterations in the epithelial cells. It was an intriguing hypothesis. Another possibility is that the normal appearing stromal cells may be derived from the epithelial lesions as a consequence of epithelial-

mesenchymal transition. Additional experiments are required to test these two possibilities. Similar to the findings of Moinfar *et al.*, Wernert *et al.* reported that frequent genomic abnormalities, such as LOH and p53 mutation, seen in cancerous epithelial cells were also present in the adjacent fibroblastic stroma. Moreover, the neoplastic epithelial cells and the adjacent fibroblasts can share the same clonalities (Wernert et al., 2001). These data suggest that either the adjacent fibroblasts are derived from the neoplastic epithelial cells or both of them are from the same progenitor cells. Nevertheless, these data indicate that the normal appearing stroma surrounding the neoplastic lesions of the breast is not normal. They may be part of the disease process and may contribute to carcinogenesis via stroma-epithelial interactions. More discussion about stromal-epithelial interactions can be found in the latter part of this introduction.

Underlying genomic instability in either cancerous epithelial cells or surrounding stroma may promote breast cancer by activating oncogenes and inactivating tumor suppressors. The significance of oncogenes and tumor suppressors in both human and murine mammary gland tumors will be discussed in detail in the subsequent sections of this introduction.

p53 and its Role in Breast Tumorigenesis

The evidence indicating p53 may be an important breast tumor suppressor stems from observations in individuals with Li-Fraumeni Syndrome. Most of these individuals possess germline mutations in *p53* and are highly susceptible to various cancers including those of the breast. Carriers who survive childhood cancer exhibit nearly 100% breast cancer penetrance (Malkin et al., 1990; Nathanson et al., 2001). Furthermore, women

who possess germline mutations in p53 pathway members such as *CHK2* (in Li-Fraumeni individuals without p53 mutations) and *ATM* (ataxia telangiectasia mutated) are also predisposed to breast cancer (Cipollini et al., 2004; Nathanson et al., 2001). In addition to germline mutations, somatic mutations in *p53* and p53 LOH are frequently defected in breast cancers (Johnson et al., 2002; Keohavong et al., 2004). Together, these data support the argument that p53 is a genuine breast tumor suppressor.

The p53 pathway is "off" in normal cells and can be turned "on" by various cellular stresses or damage via distinct mechanisms (Vogelstein et al., 2000). Cellular stress, resulting from aberrant growth signals such as active viral proteins, Ras or Myc, activates p53 through the up-regulation of ARF, a transcriptional target of E2F (Sherr and Weber, 2000). DNA damage resulting from ionizing radiation, on the other hand, activates p53 through ATM, which is phosphorylated and activated upon double strand breaks, and CHK2, which in turn is activated by ATM (Carr, 2000). Activation of p53 can also be achieved via phosphorylation by ATR (ataxia telangiectasia related) and casein II in response to a variety of other insults such as UV, chemotherapeutic agents, and protein kinase inhibitors (Meek, 1999). Despite the diverse mechanisms cells employ to turn on this pathway, the common outcome is stabilization of p53 (Vogelstein et al., 2000).

Of the numerous functions that have been attributed to p53, which include DNA repair cofactor (Wang et al., 1995) and exonuclease (Mummenbrauer et al., 1996), the most significant and intensely studied attribute of p53 is its role in transcriptional control (Ko and Prives, 1996; Levine, 1997). Virtually all p53 mutations that exist in cancer cells reduce its transcriptional activity suggesting that the transcriptional regulation of

p53 is critical for its role in tumor suppression (Vogelstein et al., 2000). Studies from *p53* knock-in mice confirm the significance of p53's transcriptional activity in suppressing tumors. The knock-in mice, carrying a transcription transactivation deficient allele of *p53* at the wild-type locus, are indeed tumor prone (Jimenez et al., 2000).

Transcriptional targets of p53 are numerous and the numbers are expected to expand even more. In general, they function in one of following categories: cell-cycle arrest, DNA repair and genome maintenance, senescence, apoptosis, and inhibition of blood vessel formation (Oren, 2003; Vogelstein et al., 2000). Defects in any of the above functions have been seen in cancer cells suggesting these functions in tumor suppression. p53 is at the hub of these massive signaling networks highlighting the critical importance of p53 in suppressing carcinogenesis.

RB, INK4, and Cip/Kip Family Members Function in Cell Cycle Control

RB family members (RB, p107, and 130) collectively regulate cell cycle progression (hence proliferation), apoptosis, and differentiation (Classon and Harlow, 2002; Sherr, 2004). Moreover, RB is also involved in regulating senescence (Classon and Harlow, 2002). Like p53, RB family members achieve much of their growth suppressive control during G1-S transition. They negatively regulate the cell cycle via modulating E2F transcription activity necessary for G1-S transition (Wang et al., 1994a), whereas $p16^{INK4a}$ enhances RB activity via inhibiting a negative inhibitor of RB activity, cyclin D/cdk4/6 (Sherr and McCormick, 2002). The p16/pRB-E2F pathway links extracellular cues to the molecular machinery that dictates the initiation of DNA replication in mammalian cells (Weinberg, 1995).

In response to mitogens during early G1 phase, D-type cyclins (D1, D2, and D3) assemble into active complexes with two cyclin dependent kinases, cdk 4 or cdk 6 (Marshall, 1999; Sherr and McCormick, 2002). Active signaling from Ras and phosphatidylinositol 3-kinase (PI3-K) in the presence of mitogens regulate cyclin D1 transcription, synthesis, and assembly with cdk4/6 and regulate the stability and nuclear retention of the cyclin D/cdk4/6 active complex (Marshall, 1999). Active nuclear cyclin D/cdk4/6 complex, in the presence of persistent mitogenic signals, co-operates with cyclin E/cdk2 complex to phosphorylate and thus inhibit RB and its family members p107 and 130. Consequently, the phosphorylation of RB relieves its growth inhibitory functions on E2Fs. E2Fs are essential transcription factors that regulate the expression of genes necessary for DNA replication and thus cell cycle progression. The hypophosphorylated RB, p107, and p130 physically interact and inhibit E2F proteins. RB primarily interacts with E2F1, 2, and 3 (Dyson et al., 1993; Lees et al., 1993) whereas p107 and p130 interact with other E2Fs such as E2F4 (Beijersbergen et al., 1994; Dyson, 1998; Ginsberg et al., 1994; Trimarchi and Lees, 2002; Vairo et al., 1995). The complexes between RB family members and various E2Fs inhibit E2F transcription activity and repress gene expression via recruiting chromatin remodeling factors such as histone deacetylases (HDACs) to E2F responsive promoters (Harbour and Dean, 2000; Ogawa et al., 2002; Rayman et al., 2002). Therefore, active hypophosphorylated RB family members negatively regulate cell cycle and block G1-S transition by repressing the transcription program mediated by E2F. Phosphorylation of RB family members by active cyclin D/cdk4/6 and cyclin E/cdk2 prevents their association with both E2F and HDACs and thus allows E2F dependent transcription to proceed (Sherr and McCormick,

2002). One of the downstream targets of E2F is cyclin E providing a positive feed back loop further activating E2F transcriptional activity (Sherr and McCormick, 2002).

$p16^{INK4a}$ modulates the RB pathway by preventing the interaction between cdk4 and cyclin D and thus inhibiting cyclin D/cdk4/6 activity (Sherr and Roberts, 1995; Sherr and Roberts, 1999). As a result, expression of $p16^{INK4a}$ inhibits downstream RB phosphorylation (Ortega et al., 2002; Roussel, 1999; Ruas and Peters, 1998; Sherr and Roberts, 1999).

$p16^{INK4a}$ belongs to the INK4 family, which includes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. Unlike $p18^{INK4c}$ and $p19^{INK4d}$, which are widely expressed during both developmental and adult tissues, the expression of $p16^{INK4a}$ is limited (Zindy et al., 1997). However, the expression of $p16^{INK4a}$ can be induced during cellular senescence (Alcorta et al., 1996; Hara et al., 1996) and environmental stress (Sherr and DePinho, 2000). Moreover, Ramirez *et al.* reported that medium conditions could also modulate p16 expression (Ramirez et al., 2001). Together, these data and the report that mice lacking $p16^{INK4a}$ are cancer prone (Krimpenfort et al., 2001; Serrano et al., 1996; Sharpless et al., 2001) suggest perhaps $p16^{INK4a}$, among all INK4 family members, is the one that counters abnormal cell proliferation signals (Sherr and McCormick, 2002).

In addition to the INK4 family, the Cip/Kip family members, including $p21^{Cip1}$, $p27^{kip1}$, and $p57^{kip2}$, also modulate the RB pathway by both promoting cyclinD/cdk4/6 complex assembly and inhibiting cyclinE/cdk2 activity. Both $p21^{Cip1}$and $p27^{kip1}$ are potent inhibitors of cyclin-E-bound cdk2 and therefore negatively regulate cell cycle progression (Blain et al., 1997; Soos et al., 1996). However, they also contribute to cyclin D1/cdk4 complex assembly, stability, and nuclear retention, and thus promote cell

cycle entry into S phase (Alt et al., 2002; Cheng et al., 1999; LaBaer et al., 1997). In quiescent cells, cyclin D levels are low and $p27^{kip1}$ is high to inhibit cyclin E/cdk2. Upon mitogenic stimulation, increased amounts of cyclin D are assembled into cdk4 and $p27^{kip1}$ complexes. The sequestering of $p27^{kip1}$ into cyclin D/cdk4 complexes relieves the cyclin E/cdk2 inhibition and allows cell cycle progression. The active cyclin E/cdk2 can further phosphorylate free $p27^{kip1}$ and trigger its degradation via ubiquitination, providing a positive feed back loop for sustained cyclin E/cdk4 activation. Upon withdrawal of mitogens, the transcription of cyclin D1 is decreased resulting in the destruction of the cyclin D1/cdk4 complex. Consequently, $p27^{kip1}$ is released from this complex and is now free to repress cyclin E/cdk2 activity, permitting re-accumulation of unphosphorylated $p27^{kip1}$. As a result, cells exit cell cycle (Sheaff et al., 1997; Sherr and McCormick, 2002; Vlach et al., 1997).

RB, INK4, and Cip/kip Family Members in Mammary Tumors

Deregulations of the RB pathway have been found in many human tumors. In fact, RB was the first tumor suppressor to be discovered as the target of a heterozygous deletion (13q14) in familial retinoblastoma patients, who rapidly develop multifocal and bilateral retinal tumors (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Subsequently, deletions in RB were found in 20-25% of breast cancer cell lines and in 7% of primary breast tumors (Lee et al., 1988; T'Ang et al., 1988).

Cyclin D1 over-expression is detected in over 50% breast cancer cases (Sherr and McCormick, 2002) and is also seen in up to 50-87% of early stage breast lesions such as atypical ductal hyperplasia and ductal carcinoma in situ (DCIS) suggesting an early role

of cyclin D1 dysregulation in breast cancer progression (Gillett et al., 1998; Simpson et al., 1997; Weinstat-Saslow et al., 1995). Gene amplification is a common mechanism for cyclin D1 over-expression. Approximately, 20% of breast cancers exhibit cyclin D1 gene amplification (Dickson et al., 1995). Moreover, over-expression of cyclin D1 in breast tumors correlates with high tumor grade (Fernandez et al., 1998).

Interestingly, in contrast to tumors caused by inactivation of RB or over-expression of cyclin E, many tumors that over-express cyclin D1 do not express high levels of cyclin E (Zukerberg et al., 1995), a canonical E2F target gene, and do not exhibit increased proliferation (Oyama et al., 1998). These data suggest that additional functions of cyclin D1 different from its role in cdk-dependent and E2F-mediated cell cycle promotion may be the oncogenic activity of cyclin D1 in these tumors. In support of this hypothesis, emerging data from Lamb *et al.* indicate that cyclin D1 may also contribute to breast cancer or any other malignancy via a cdk kinase independent role as a direct transcriptional factor, in addition to its traditional role as positive cell cycle regulators including both cdk kinase dependent inhibition of RB and titration of cell cycle inhibitors such as p21 and p27 away from cyclin E/cdk2 (Ewen and Lamb, 2004; Lamb et al., 2003). To dissect out the cdk dependent and in-dependent functions of cyclin D1, Lamb *et al.* introduced both wild-type and a cyclin D1 mutant (K112E) that are unable to activate cdk4 (Hinds et al., 1994) into MCF-7 human mammary epithelial cells (Lamb et al., 2003). Employing microarray experiment, they found that the cdk independent mutant of cyclin D1 is able to up-regulate many genes that are up-regulated by wild type cyclin. Furthermore, through data mining using multiple independent databases from more than 500 human tumors and cancer cell lines and biochemical experiments, they

found that C/EBPβ, a transcription factor that has been implicated in breast cancers (Zahnow, 2002), is involved in regulating genes that are affected by cyclin D1 over-expression. Perhaps the cdk kinase independent oncogenic effect of cyclin D1 is achieved trough the interplay between cyclin D1 and C/EBPβ.

Cyclin E is also over-expressed in a subset of breast cancers (Nielsen et al., 1996; Nielsen et al., 1997). In addition to cyclin E over-expression, a variant of cyclin E, which forms constitutively active complex with cdk2 throughout the cell cycle and results in deregulated cell cycle, exists in breast cancer cells (Keyomarsi et al., 1995). Furthermore, amplification of cyclin E's partner, cdk2 that form the active complex with cyclin E, has also been reported in human cancers including ovarian and colorectal tumors (Kitahara et al., 1995; Marone et al., 1998).

The link between $p16^{INK4a}$ and human cancers was established after the observation that it is located in the locus (*CDKN2a*) frequently deleted in many human cancers (Kamb et al., 1994a; Nobori et al., 1994). Furthermore, individuals with germline point mutations in $p16^{INK4a}$ are highly susceptible to familial melanoma (Gruis et al., 1995; Hussussian et al., 1994; Kamb et al., 1994b). Loss of $p16^{INK4a}$ is also detected in about 30% of breast cancers (Sherr and McCormick, 2002) and in 40-60% of breast cancer cell lines (Kamb et al., 1994a). Reintroducing $p16^{INK4a}$ into breast cancer cell lines causes a dose-dependent cell cycle arrest (Craig et al., 1998; Foster et al., 2001).

Somatic inactivation of $p16^{INK4a}$ can be the result of both genetic (point mutation and deletion) and epigenetic (promoter hypermethylation) alterations (Esteller et al., 2001; Merlo et al., 1995; Ruas and Peters, 1998; Sharpless and DePinho, 1999). Furthermore, aberrant cytoplasmic expression of p16 is also seen in 9% of invasive (n=

368) and 4% of intraductal carcinomas (n=52) (Emig et al., 1998; Geradts and Wilson, 1996). The importance of $p16^{INK4a}$ in human cancers was further strengthened by the finding that alterations in cdk4 (either point mutations that disrupt the binding of $p16^{INK4a}$ and render it $p16^{INK4a}$ resistant or gene amplification) and cdk6 (over-expression) are also detected in human cancers (Tang et al., 1999; Wolfel et al., 1995; Zuo et al., 1996).

Under-expression or mutational inactivation of additional CDK inhibitors also exists in breast cancer. A reduction in p27 expression is frequently seen in breast cancer and correlated with aggressive phenotypes and poor prognosis (Blain et al., 2003; Catzavelos et al., 1997; Tan et al., 1997). Similarly, both *p21* mutations that impair its ability to inhibit CDKs (Balbin et al., 1996) and the localization of p21 in the cytosol are associated with poor prognosis (Winters et al., 2003) and have been observed in breast cancer cells. However, to add to the complexity, over-expression of CDK inhibitors such as p21 may also promote some early stage neoplasias (Biankin et al., 2001). Given that p21 also facilitates cyclin D/cdk4/6 assembly and activation in addition to its role in cyclin E/cdk2 inhibition, this observation may not be so surprising.

Connection between p53 and RB

One of the consequences of RB inhibition and subsequent E2F activation is the up-regulation of a tumor suppressor, ARF, the alternative reading frame product of INK4a locus that also encodes tumor suppressor $p16^{INKa}$ (Quelle et al., 1995). The ARF protein directly binds to and sequesters HDM2, a negative regulator of p53, in the nucleolus of the cell. Furthermore, ARF also antagonizes HDM2's E3 ubiquitin ligase

activity thereby preventing p53 degradation and triggering a p53-dependent cell cycle arrest or apoptosis (Lowe and Sherr, 2003; McClatchey and Jacks, 1998; Sherr and McCormick, 2002). The cell cycle arrest in response to p53 activation is mediated by the up-regulation of p21, which modulates the RB pathway via inhibiting cyclin E/cdk2, providing a negative feed back loop and highlighting the complexities of cellular pathways.

BRCA1 and BRCA2 in Breast Cancer

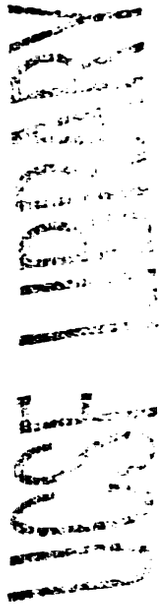
Approximately, 8-10% of breast cancer cases are heritable indicating the genetic aspect of the disease. Individuals who possess germline mutations in BRCA1 or BRCA2 are predisposed to breast and ovarian cancers indicating the tumor suppressing function of the two proteins. The estimated lifetime breast cancer risk for those who carry germline mutation in BRCA1 varies from 36% to 85% depending on the populations used for the analysis (Nathanson and Weber, 2001). The median age of diagnosis in these individuals is 42 years (Nathanson et al., 2001). The lifetime breast cancer risk for BRCA2 mutation carriers is similar to that of BRCA1 carriers, but at a later age of onset (Welcsh and King, 2001). Interestingly, unlike BRCA1, male carriers for BRCA2 have an ~ 6% lifetime breast cancer risk, which is 100 fold higher than the general population (Nathanson et al., 2001).

Both *BRCA1* and *2* encode two large proteins. They interact with numerous molecules and are involved in many fundamental cellular activities. Clues for their cellular functions stem from the observations that *BRCA1* or *2* mutant cells lack genomic integrity and accumulate chromosomal abnormalities including DNA breaks, aneuploidy,

and centrosome amplification (Deng and Scott, 2000; Welcsh and King, 2001). Subsequent experiments demonstrated that the physiological functions of BRCA1 in maintaining genomic integrity are extremely complex. BRCA1 is essential in many cellular functions including DNA damage sensing and repair, recombination and DNA replication, transcription-coupled repair, chromatin remodeling, cell cycle checkpoint control, protein ubiquitination (E3 ligase activity), and apoptosis (Powell and Kachnic, 2003; Venkitaraman, 2002). BRCA 2 has also been implicated in maintaining genomic integrity, chromatin remodeling, transcriptional activation, and RAD51-associated recombination (Powell and Kachnic, 2003; Venkitaraman, 2002).

Interestingly, despite the fact that both BRCA1 and 2 are ubiquitously expressed and function in cellular pathways that are fundamental to all cells, individuals who carry the germline mutations in these two genes almost specifically develop breast or ovarian cancers (Welcsh and King, 2001). Recent data from David Livingston's laboratory point to a possible molecular mechanism for this gender and tissue specificity in BRCA1 cancers (Ganesan et al., 2004; Ganesan et al., 2002). They found that a fraction of BRCA1 is associated with Xi-specific RNA transcripts (XIST), which coat the X chromosome in *cis* and initiate a cascade of chromatin modifications on the X chromosome, and hence trigger X chromosome inactivation (Heard, 2004). Both cultured cells and cells directly isolated from BRCA1-/- primary tumors lacking BRCA1 exhibit defects in XIST localization. Consistently, Jazaeri *et al.* reported that ovarian cancer cells deficient in BRCA1 over-express some X chromosomal genes (Jazaeri et al., 2002). Acute down regulation of BRCA1 in female diploid cells also leads to defects in Xi and re-expression of GFP inserted on Xi whereas reconstitution of wild-type BRCA1

in cells lacking BRCA1 rescues the Xist localization phenotype. Taken together, these data suggest that BRCA1 may be required for proper formation and/or maintenance of Xi. However, the significance of BRCA1 in Xi formation and/or maintenance in suppressing tumorigenesis remains unanswered. It will be interesting to find out whether re-activation of the X chromosome can initiate and/or facilitate the tumorigenic process in mammary and ovarian cells. It is nevertheless appealing to speculate that re-activation of some (or all) X chromosomal genes in BRCA1 -/- cells specifically contribute to breast and ovarian tumor development. In support of this idea, gain of X chromosome and/or loss of Y chromosome among other genomic abnormalities are commonly seen in male breast cancer (Rudas et al., 2000; Teixeira et al., 1998). Furthermore, men with Klinefelter's syndrome, who possess increased X dosage due to XXY genotype, are predisposed to breast cancer arguing for a role of increased X dosage in promoting breast cancer development (Swerdlow et al., 2001). Moreover, hormones might also play a role in facilitating the tissue specific cancer development in the carriers.



Genetic Modifying Loci for BRCA Mutation in Breast Cancer

Substantial variation of breast cancer penetrance in BRCA 1 or 2 carriers suggest that modifying loci affecting BRCA carrier's breast cancer risk exist. In an attempt to discover the specific breast cancer modifying loci in *BRCA* carriers, Nathanson *et al.* carried out linkage analysis between three loci that are frequently lost in BRCA1 breast tumors as detected by array CGH (Tirkkonen et al., 1997) and breast cancer risk. Significant linkage between loci at chromosome 5q 33-34, which is lost in 86% BRCA1 breast cancers (Tirkkonen et al., 1997), and *BRCA1* mutation in breast cancers has been

found suggesting that this locus may be a modifier specific for breast cancer penetrance in kindreds with *BRCA1* germline mutation (Nathanson et al., 2002). In addition, androgen receptor alleles that contain increased CAG repeats and an allele of AIB1 (transcriptional co-activators for steroid hormones in estrogen-dependent transcription) that contains increased polyglutamine repeats have been found to be associated with increased breast cancer risk in individuals carrying germline *BRCA1* mutations (Nathanson and Weber, 2001).

Connection Between BRCA1 and $p16^{INK4a}$-cyclin D/cdk4/6-RB-E2F Pathway

RB-E2F pathway plays a central role in regulating the expression of numerous genes that promote DNA synthesis, cell-cycle progression, apoptosis (both p53-dependent and independent), and activation of p53 tumor suppressor through up-regulation of ARF. In addition to p53, the link between the $p16^{INK4a}$-cyclinD/cdk4/6-RB-E2F pathway and another famous tumor suppressor, BRCA1, has also been reported. Wang *et al.*, noted that the *BRCA1* promoter contains E2F binding sites and the expression of BRCA1 is dependent on the status of the $p16^{INK4a}$-cyclinD/cdk4/6-RB-E2F pathway (Wang et al., 2000). Ectopic expression of E2F, cyclin D1 and cdk4 up-regulate BRCA1 expression where as $p16^{INK4a}$ and RB repress BRCA1 expression. The functional significance of this link remains to be fully clarified. Thus far, the existing data suggest that one function of BRCA1 up-regulation in response to deregulated RB-E2F pathway may be to promote apoptosis. It has been shown that BRCA1 can directly bind to p53 and stimulate p53-dependent transcription of pro-apoptotic genes (Ouchi et al., 1998;

Zhang et al., 1998a). Furthermore, BRCA1 can promote p53-independent apoptosis through p53-independent activation of GADD45, which can activate JNK/SAPK and induce apoptosis (Harkin et al., 1999). Thus, one of the tumor suppressing activities of BRCA1 may come from its ability to oppose the aberrant proliferation signals from deregulated RB-E2F pathway by activating the apoptosis response.

Other Breast Cancer Susceptibility Genes

Even though together BRCA 1 and 2 are accounted for nearly all breast and ovarian cancer families with autosomal dominant inheritance of susceptibility and the families with four or more breast cancer cases at an early age onset of 60 or less, they only account for a small fraction of hereditary breast cancers. Additional genetic loci must exist to confer either increased or decreased breast cancer susceptibilities. Nearly a decade after the momentous discovery of the breast cancer predisposition genes *BRCA 1* and *2* (Hall et al., 1990; Miki et al., 1994; Wooster et al., 1995; Wooster et al., 1994), the search for additional breast cancer susceptibility genes has yielded limited results (Nathanson and Weber, 2001). Loci implicated as additional breast cancer susceptibility genes include *STK11/LKB1* (a serine-threonine kinase), *Stk6/STK15, APC, PTEN, p53, ATM*, and *CHEK2* (Consortium, 2004; Deng and Brodie, 2001; Ewart-Toland et al., 2003; Meijers-Heijboer et al., 2002; Nathanson et al., 2001). In addition, allelic variations in androgen receptor and AIB1 may also contribute to increased breast cancer penetrance in BRCA 1 carriers (Nathanson and Weber, 2001). Despite the success in identifying these breast cancer susceptibility genes, many inherited breast cancer cases still remain to be unaccounted for. High-density genome wide analysis such as SNP

microarray may be of use to search for breast cancer susceptibility genes with either high or low penetrance in the future (Houlston and Peto, 2004).

Given the complexity of breast cancers, appropriate model systems are necessary for dissecting the pathways in breast cancer initiation, progression, maintenance, and invasion. In the next part of this introduction, I will describe the model systems currently employed for investigating the disease and summarize the knowledge gained from these studies.

Knowledge Gained from Studying Breast Cancer Genetics in Murine Models

Studies of cancer genetics in murine models have been extremely fruitful thanks to the extensive genetic tools available and the easy manipulation and monitoring of the laboratory animals. For human breast cancer research, mouse models can be classified into three main categories: 1) chemical and virus induced, or ionizing radiation- induced mice tumor model, 2) genetically engineered mice (GEM) such as transgenic, knockouts, knockins, and conditional Cre/LoxP or Flp/Frt recombination based transgenic and knock out/ins, 3) xenoplants models which include heterotopic, such as subcutaneous or kidney capsule transplant, and othotopic, such as mammary fat pad transplant, tumor models (Cardiff et al., 2000; Kim et al., 2004a; Tuveson and Jacks, 2002; Wagner, 2004). Studies from each of these model systems have provided invaluable insight into breast cancer initiation and progression. In addition, the murine model is effective at dissecting

the hormonal effects on breast cancer development. However, the hormonal studies are beyond the scope of this introduction.

Oncogenes in Breast Cancer Development

Transgenic mice expressing various oncogenes have been produced to study the role of variety oncogenes in mammary tumor initiation and progression. Significant improvement in transgenic studies came with the ability to manipulate transgene expression specifically in the mammary gland. Stewart *et al.* describe the first generation of mammary tumors in transgenic mice with MTV/*myc* fusion genes in 1984 (Stewart et al., 1984). The murine mammary tumor virus (MMTV) promoter includes hormone-regulated enhancer elements, and thus the expression of the transgene is dependent on the hormonal status, which changes during murine embryogenesis, pregnancy and lactation. (Jamerson et al., 2004; Kim et al., 2004a). They showed that even though the expression of *c-myc* in the mammary gland had no obvious effect during early development, it did contribute to the formation of mammary adenocarcinomas. Furthermore, the F1 progeny that inherit the MTV/*myc* transgene also develop mammary adenocarcinomas. This data indicate that deregulated c-myc expression in mammary gland tissues acts as a heritable and predisposing factor in mammary tumorigenesis (Stewart et al., 1984).

Since the study by Stewart *et al.*, the use of transgene expression specifically in murine mammary gland under the control of MMTV promoters has been widely applied to study the functions of various oncogenes such as *wnt* (Li et al., 2000), *her2/Neu, myc* (Guy et al., 1992; Jamerson et al., 2004; Sinn et al., 1987), *H or N-ras* (Mangues et al., 1990; Sinn et al., 1987), *c-src* (Webster et al., 1995), *TGF-α* (Matsui et al., 1990), *cyclin*

D1 (Wang et al., 1994b), *Fgf3* (Muller et al., 1990), and *COX-2* (Liu et al., 2001). Transgene expression of each of these oncogenes except *Fgf3* leads to mammary gland carcinogenesis generating focal or multifocal mammary lesions. The focal occurrence of the mammary tumors suggests multi-step carcinogenesis since all mammary epithelia expressed oncogenes, but only few progress to become malignant. Additional alterations may be required for tumorigenesis (see next paragraph for examples of combined effects of two or more oncogenes). MMTV-*Fgf3* mice develop early lesions such as mammary hyperplasia but rarely tumors (Muller et al., 1990) providing a good *in vivo* model for studying early steps of breast carcinogenesis.

The murine whey acidic protein (WAP, a normal protein component of milk) promoter is another popular and highly utilized promoter that directs the expression of specific oncogenes within the secretory mammary epithelium of mice during late-pregnancy and lactation (Jamerson et al., 2004). Schoenenberger *et al.* reported that WAP/*c-myc* transgene expression was sufficient to induce mammary tumors (Schoenenberger et al., 1988). Significantly, WAP-*stromelysin*-1 transgenic mice also develop mammary tumors (Sternlicht et al., 1999). Stromelysin -1 is an extra cellular matrix remodeling protein. The results from WAP- *stromelysin* suggest that alterations in the microenvironment can contribute to mammary gland carcinogenesis. Indeed, many studies have pointed to a facilitating role of the microenvironment in carcinogenesis (Coussens et al., 2000; Lin and Pollard, 2004; Olumi et al., 1999; Tlsty and Hein, 2001).

In addition to MMTV and WAP, a variety of other promoters including MT, BLG, C3(1), UASXGAL4 bitransgenic, MMTV/MT bitransgenic, RSV, and H4 are also employed to target gene expressions to the mammary gland (Cardiff et al., 2000). The

more sophisticated, inducible and reversible MMTV-*rtetTA*/tetOP promoter has been developed to regulate transgene expression such as for *c-myc* (Jamerson et al., 2004).

One major disadvantage for using either MMTV or WAP as promoters to drive transgenic expression is that both promoters are hormone sensitive. Hence, it is difficult or impossible to address hormonal effects that may interact with the transgenic activities in the mammary tumorigenic process (Blackburn and Jerry, 2002).

Contributions of combinatorial effects of two or more oncogenes have also been tested specifically in murine mammary gland tumor formation (Jamerson et al., 2004). For example, *c-myc* single transgene expression under the MMTV promoter in murine mammary glands leads to mammary tumors with a 50% incidence following a 7-14 month latency in virgin mice (Jamerson et al., 2004; Schoenenberger et al., 1988; Stewart et al., 1984). Similarly, MMTV-*H-ras* transgenic mice develop mammary adenocarcinomas with a 50% incidence following an average of 186 day latency in virgin female mice (Sinn et al., 1987). In *c-myc* and *H*-ras bitransgenic mice, mammary tumors emerge in 46 days in contrast to 186 days in *H-ras* mice and 7-14 month in *c-myc* mice. In addition to the reduced latency in the bitransgenic female mice, tumor incidence in male mice is also elevated (Sinn et al., 1987). Hence, *c-myc* and *H-ras* co-operate in the mammary tumorigenesis as evidenced by the significant synergistic effect seen in the bitransgenic mice (Sinn et al., 1987). c-myc expression sensitizes cells to apoptosis. But in the bitransgenic mice, apoptosis was reduced (Sinn et al., 1987). The co-operative effect or synergy between *H-ras* and *c-myc* may be explained by this reduction in apoptotic index in the bitransgenic mice (Hundley et al., 1997). Interestingly, despite the co-expression of two oncogenes in mammary tissues, mammary tumors still arose in

focal lesions in the bitransgenic mice after a latent period. This stochastic pattern in mammary tumor formation suggests that yet additional unidentified molecular alterations are required for the full transformation (Sinn et al., 1987).

An effort was subsequently made to generate tritransgenic mice expressing *c-neu*, *c-myc*, and *H-ras*. Tumor incidence was increased to 100% and tumor latency was significantly reduced in the tritransgenics (Cardiff et al., 1991). In these experiments, tumor incidences for *myc*, *ras*, *neu*, *myc/neu*, and *myc/neu/ras* were 20%, 67%, 73%, 91%, and 100% respectively. Tumor latencies were roughly 299 days for *c-myc*, 263 days for *ras*, 228 days for *neu*, 141 days for *myc/neu*, and 72 days for *myc/neu/ras* (Cardiff et al., 1991; Jamerson et al., 2004). Moreover, tumor phenotypes in these tritransgenic mice are similar to the large cell/dense glandular adenocarcinoma phenotype seen in MMTV-*c-myc* transgenic mice. This result suggests that *c-myc* plays a dominant role in the oncogenic transformation in combination with *c-neu* and/or *H-ras*.

Despite the variations among different experiments, studies in transgenic mice clearly demonstrate a co-operative role of multiple oncogenes in mammary tumor development. Similar requirements of multiple oncogenes for complete transformation are also seen in human mammary epithelial cells. Elenbass *et al.* presented data indicating that transformation of human mammary epithelial cells in culture requires SV 40 Large T antigen, the telomerase catalytic subunit, and H-ras (Elenbaas et al., 2001).

The Role of p53 in Suppressing Mammary Gland Tumor Development

The roles of tumor suppressors in suppressing mammary gland tumors have been examined using knockout mice. p53 and pRB are the two most frequently mutated

proteins in human cancer demonstrating their normal function in inhibiting tumor formation (Sherr and McCormick, 2002). A large body of evidence from knockout studies clearly demonstrates tumor suppressor role of these molecules (Donehower et al., 1995a; Donehower et al., 1992; Harvey et al., 1993; Jacks et al., 1992; Jacks et al., 1994; Purdie et al., 1994; Williams et al., 1994).

Most mice with no p53 tumor suppressor activity are viable and survive to adulthood except ~ 10% p53 -/- mice, which die of exencephaly (Armstrong et al., 1995; Jacks, 1996; Sah et al., 1995). However, p53-/- animals are highly susceptible to tumor formation and die before 6 months of age (Donehower et al., 1992). They spontaneously develop predominantly lymphomas and soft tissue sarcomas (Donehower, 1996; Donehower et al., 1992; Jacks et al., 1994). Mammary tumors, on the other hand, are rarely seen in the p53 -/- or p53 +/- mice with a mixed genetic background (75% C57BL/6 and 25% 129/Sv) or 129/Ola background (Donehower, 1996; Donehower et al., 1992; Jacks et al., 1994; Purdie et al., 1994). These observations are in contrast with the observations in humans with Li-Fraumeni syndrome. Approximately half the individuals with Li-Fraumeni syndrome possess germline mutations in *p53* and they develop pre-menopausal breast cancer and other malignancies with high frequency (Kleihues et al., 1997; Malkin et al., 1990; Srivastava et al., 1990).

The lack of mammary tumors or other adenocarcinomas in p53 -/- mice raise several possibilities. The first possibility is that p53 is dispensable in murine mammary tumor initiation or progression. The fact that no mammary tumors were observed in either p53-/- or p53 +/- mice seems to suggest this possibility. In addition, DMBA-induced mammary tumors in p53 +/- mice do not lose the wild-type allele (Jerry et al.,

1994). However, it is unlikely that p53, a major tumor suppressor, is dispensable in mammary carcinogenesis. Even though p53 -/- mice don't form mammary tumors, serially transplanted mammary cells from either p53 null or heterozygous mice frequently develop ductal hyperplasias and ductal carcinoma in situ (DCIS) lesions that eventually progress into invasive breast tumors (Jerry et al., 2000; Medina et al., 2002). The presence of pituitary isografts, which provide continuous hormonal stimulation, increased tumor frequency from 60 to 100% and reduced tumor latency from 50 to 37 weeks in studies using theses serially transplanted mammary cells (Jerry et al., 2000).

A second possibility is that p53 is an important tumor suppressor in mammary carcinogenesis, however, the rapid and rampant development of lymphomas and sarcomas in p53 null mice obfuscate the phenotypical changes in the epithelial cells including mammary epithelium. The result from a serially transplant experiment, in which p53 -/- or +/- mammary epithelial was transplanted into the p53 wild type BALB/c female mammary fat pad, points to a role of p53 in suppressing mammary tumor formation (Jerry et al., 2000; Medina et al., 2002). The conditional p53 null or heterozygous in mice mammary gland will clearly address this second possibility and clarify p53's contribution in suppressing mammary carcinogenesis. Similarly, specific over-expression of dominant-negative p53 mutants under the WAP or MMTV promoters in the murine mammary gland is also useful to elucidate p53's function in mammary gland.

p53 functions as a tetrameric protein and the presence of a dominant negative mutant would disrupt the proper tetramerization that's required for its transcriptional activity (Jeffrey et al., 1995). Mutant p53, *p53-R172H*, functions as such a dominant-

negative mutant and its human equivalent (*R175H*) is the second most commonly found mutation in human breast cancers (Hainaut et al., 1997). The expression of *p53 R172H* specifically in mammary glands leads to increased tumor burden and decreased tumor latency in the presence of carcinogens (Li et al., 1998) indicating the tumor suppressor role of p53 in mammary tumors.

A third possibility is that genetic modifiers in different murine genetic backgrounds influences p53's ability in suppressing mammary tumors. In contrast to C57BL/6x129/Sv mice, from which the original p53-/- mice were generated (Donehower et al., 1992), BALB/c mice have been shown to be sensitive to radiation-induced mammary tumors (Ponnaiya et al., 1997; Ullrich et al., 1996). To address whether genetic modifiers or low penetrance susceptibility genes (Balmain, 2002) in different genetic backgrounds influence p53's tumor suppressor function, Kuperwasser *et al.*, crossed the p53 -/- into the BALB/c background (Blackburn et al., 2003; Kuperwasser et al., 2000). In this BALB/c background, p53 -/ - mice still primarily die of lymphomas with a median survival time of 15.4 weeks. However, a different result was obtained in p53 heterozygous BALB/c mice. About 55% p53 +/- BALB/c mice developed mammary tumors with a 54 weeks latency. In addition, the mammary tumors, which arose from p53 +/- BALB/c mice, frequently lost the remaining wild type p53 allele indicating a selective pressure to lose p53 activity. Furthermore, the tumor spectrum observed in p53 +/- BALB/c female mice are similar to the tumor spectrum seen in female individuals with Li-Fraumeni syndrome (Akashi and Koeffler, 1998; Deng and Brodie, 2001; Kleihues et al., 1997; Kuperwasser et al., 2000; Malkin et al., 1990). Hence, the p53 +/-

BALB/c mice model may function as invaluable model system for studying breast carcinogenesis in individuals with Li-Fraumeni Syndrome.

The apparently different tumor spectra in p53 +/- mice with either BALB/c or C57BL/6x129/Sv backgrounds suggest the existence of genetic modifiers in different inbred mouse lines (Donehower et al., 1995b). The BALB/c mice may possess modifying loci that increase their susceptibility to mammary tumors. Intriguingly, the BALB/c laboratory mice contain a polymorphic $p16^{INK4a}$ allele and a polymorphic *DNA-PK* allele, both of which contain two point mutations (Yu et al., 2001b; Zhang et al., 1998b). The two point mutations in *DNA-PK* result in increased radiation-induced genomic abnormalities in mammary epithelial cells and thus may render increased susceptibility to radiation-induced mammary tumors in BALB/c mice. The two point mutations in $p16^{INK4a}$ reduce the ability of $p16^{INK4a}$ to inhibit Rb phosphorylation, and thus render the polymorphic $p16^{INK4a}$ allele hypomorphic (Herzog et al., 1999; Zhang et al., 1998b; Zhang et al., 2001). The function of $p19^{ARF}$, on the other hand, does not appear to be affected (Zhang et al., 1998b; Zhang et al., 2001). Studies with melanoma patients who possess mutations in *CDKN2A* gene, which encode p16, suggested that these individuals are at greater risk in developing breast cancer (Borg et al., 2000; Plna and Hemminki, 2001). These observations raise the question whether hypomorphic $p16^{INK4a}$ alleles modify the tumor incidence and spectrum in p53 +/- BALB/c mice. Perhaps, deficiency in p16 activity co-operates with deficiency in p53 function to promote mammary carcinogenesis. Studies reported by Sharpless *et al.* seem to argue against this possibility of co-operation between p16 and p53 deficiency in mammary carcinogenesis (Sharpless et al., 2002). Sharpless *et al.* examined mice (mixed genetic background;

~87.5% FVB/n) with p53 and/or p16 deficiency for tumor incidence and spectrum in different tissues. While they found that p53 and p16 double null mice did develop tumors earlier, they did not develop mammary carcinomas. The increased tumor frequency and reduced tumor latency in mice missing both p53 and p16 functions suggest that p53 does indeed co-operate with p16 to prevent tumor formation. But the absence of mammary tumors in p53 and p16 double null mice seems to argue against the role of p16 in modifying p53 function during mammary carcinogenesis. These results suggest that additional modifier loci other than $p16^{INK4a}$ may explain the mammary tumor susceptibility in the BALB/c background. However, the limited sample size in their study prevents definitive conclusions.

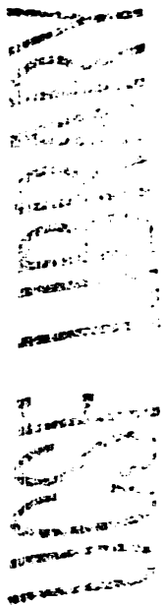
In an attempt to find the genetic modifiers in BALB/c mice that explain their mammary tumor susceptibility, Blackburn et al. crossed BALB/cMed-*Trp53*-/- male mice with C57BL/6J-*Trp53* +/+ mice to generate p53 heterozygous mice with mixed genetic background. The F1 females (C57BL/6J xBALB/cMed-*Trp5*+/+) from this cross were further crossed with BALB/cMed- *Trp53*-/- males to generate p53 heterozygous N2 with (C57BL/6xBALB/c)xBALB/c mixed genetic background (Blackburn et al., 2003). They found that diminishing BALB/c background with the introduction of C57BL/6 alleles increases murine survival and tumor latency and decreases tumor frequency. Furthermore, tumor spectra are also altered with the introduction of C57BL/6 alleles into the BALB/c background (Blackburn et al., 2003). Taking the candidate gene approach, Blackburn *et al.*, found that neither BALB/c alleles of *Prkdc* or *Cdkn2a,* which encode DNA-PK and p16 respectively, alone statistically modified p53 alleles in mammary tumor development. However, when the interactions between these two loci are

considered, both alleles do modify tumor incidence, latency, and spectra in p53 heterozygous mice with *Cdkn2a* having the stronger effect (Blackburn et al., 2003). This result demonstrates compound interactions between modifying genes and highlight the complexity of genetic interactions in determining mammary tumor phenotypes (Blackburn et al., 2003; Blackburn et al., 2004). To add to the complexity, data reported by Blackburn *et al.*, also suggest that the effects of multiple modifying loci are also likely to be cell type, cell cycle, and carcinogen specific (Blackburn et al., 2003).

The fourth possibility is that down regulation of p53 in the mammary gland creates a conditional phenotype. Under permissive conditions, p53 -/- mammary cells will embark on the tumorigenic process and unmask a role for p53 in mammary carcinogenesis. p53 is an important negative regulator of the cell cycle and thus serves as cell cycle checkpoint protein (Hofseth et al., 2004). Conditional phenotypes have been exemplified in many cell cycle checkpoint proteins including p53. Cell cycle checkpoint controls (CCCC) function as surveillance to ensure the accurate duplication and segregation of genetic material during each cell division and to prevent the persistence of genomic abnormalities (Hartwell and Weinert, 1989; Weinert and Hartwell, 1988). Under certain conditions such as in the presence of DNA damage, cell cycle checkpoint control is activated to halt the cell cycle (Hartwell and Weinert, 1989; Myung et al., 2001; Weinert and Hartwell, 1988). Thus, mutations in cell cycle checkpoint control proteins may only manifest the phenotypes when such conditions exist.

One of the conditions that permit manifestation of tumor phenotypes in p53 null mice in mammary epithelium may be telomeric dysfunction, a consequence of ever shortening telomeres. Unlike humans where the average telomere length is ~10 kb,

telomeres in mice are ~ 30-40 kb (Newbold, 1997). Furthermore, adult murine somatic tissues express telomerase in contrast to their human counterparts (Greenberg et al., 1998). Telomerase is a ribonucleoprotein complex that possesses RNA dependent DNA polymerase activity (Chan and Blackburn, 2004). The primary function of telomeres (at least the current view) is to maintain telomere length by synthesizing new DNA repeats at the telomere using its internal RNA template. To generate a mouse model that will better mimic the progressive telomere shortening phenotype in humans, Artandi *et al.* mutated the *Terc* RNA subunit of telomerase in mice and thus knocked out telomerase activity (Artandi et al., 2000). In the absence of telomerase activity, proliferating murine cells undergo progressive telomere shortening similar to proliferating human cells and reach a critically short telomere length that instigates telomeric dysfunction after several generations of breeding the *Terc* RNA null animals (Artandi et al., 2000). Importantly, the lack of telomerase function and/or the existence of shortened telomeres in the late-generation *Terc* -/- mice drastically changes both the tumor frequency and spectrum of p53 heterozygous mice (Artandi et al., 2000). In these mice, increased frequency of mammary carcinomas was observed along with increased frequency other carcinomas. This data indicate that deficiency in p53 activity in the context of shortened telomeres and/or absence of telomerase activity permits mammary tumor development. Perhaps the genomic abnormalities resulting from shortened telomeres and/or the absence of telomerase activity create a damage condition that requires proper p53 activity to prevent cell cycle progression. In other words, p53 is indeed a tumor suppressor in murine mammary carcinomas. However, its function in modulating mammary tumorigenesis is only revealed under conditions such as shortened telomeres in mice.



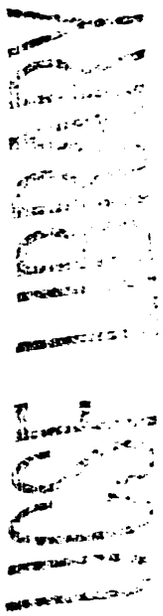
Haploinsufficiency of p53

The haploinsufficiency of p53 function in suppressing tumors has been demonstrated in numerous experiments (Chandar et al., 2000; Jones et al., 1997; Venkatachalam et al., 1998; Venkatachalam et al., 2001). Donehower *et al.* and Purdie *et al.* have reported that only ~50% tumors in p53 heterozygous mice exhibited LOH at the wild type p53 allele. The fact that some tumors that arose in p53 heterozygous mice still retain a functional wild type p53 allele indicates that reduction of p53 dosage can promote cancer development (Venkatachalam et al., 1998).

The reduction in p53 dosage in the heterozygous mice has been shown to result in an intermediate level of p53's transcriptional activity, growth control, and apoptosis phenotype when compared to the both wild type and p53 null cells (Colombel et al., 1995; Gottlieb et al., 1997; Venkatachalam et al., 2001). Studies on co-operativity between heterozygous p53 and various other tumorigenic mutations also revealed that reduction in p53 dosage is sufficient to promote tumors and reduce tumor latency (Kemp et al., 1993; Williams et al., 1994). In a chemically-induced skin tumor model, reduction in p53 dosage promotes malignant progression (Kemp et al., 1993). Interestingly, this reduction in p53 dosage does not affect tumor initiation. These results, and others, expose the complexity of p53 activity in different cell types and under different conditions.

Transcriptional Transactivation Activities of p53 are Necessary for Suppressing Tumor Formation

To address whether the transcriptional transactivation activity of p53 is required to suppress tumorigenesis, Jimenez *et al.* replaced the wild-type *p53* allele with a mutant allele (encoding changes at Leu25 and Trp26) known to be essential for transcriptional transactivation and Mdm2 binding (Jimenez et al., 2000). The resulting knock-in mice with mutant alleles of *p53* are also tumor prone and phenotypically similar to p53 null mice, suggesting the p53 tumor suppressor functions in mice require the transcriptional transactivation activity. Through studies of various p53 mutants and their abilities in growth suppression in tumor cell lines, Pietenpol *et al.* reached the same conclusion: i.e., the sequence specific transcriptional transactivation activity of p53 is required for its function as a tumor suppressor (Pietenpol et al., 1994).



A prominent transcriptional target of p53 is the cyclin E-cdk2 inhibitor, $p21^{Cip1}$. Interestingly, even thought $p21^{Cip1}$mediates many aspects of p53-induced growth arrest (Brugarolas et al., 1995; Deng et al., 1995), $p21^{Cip1}$-/- mice do not phenocopy p53 -/- mice and are not tumor prone (Deng et al., 1995). This result suggests that the tumor suppression function of p53 is not mediated through $p21^{Cip1}$ alone, and additional transcriptional targets, such as those involved in apoptosis, of p53 are required. Intriguingly, Rowan *et al.* reported that p53's ability in inducing apoptosis may be more important than its cell cycle arrest properties in suppressing tumor development (Rowan et al., 1996).

Numerous experiments have clearly demonstrated the important role of p53 in suppressing mammary tumorigenic process. The co-operation between p53 and various tumor suppressors or oncogenes have also been under intense investigation. While co-

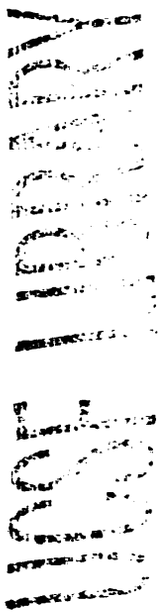
operation between various oncogenes or tumor suppressors and p53 exist in the mammary tumorigenic process, there is no apparent co-operation between *c-myc* and p53 in mammary tumor development. Interestingly, p53 and *c-myc* co-operation does exist in tumors other than mammary tumors, such as in lymphomas (Elson et al., 1995). These data suggest that co-operativity between *c-myc* and p53, and likely others, are tissue or cell type specific.

The Importance of an Intact RB Pathway in Suppressing Tumorigenesis

Knockout mice modified in another important tumor suppressor, RB, or its family members, have also been generated (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). RB -/- embryos die between day 13 and 15 from deficient erythropoiesis, defective differentiation, excessive proliferation, and cell death in multiple organs, whereas RB heterozygous mice develop pituitary and thyroid tumors, but not retinoblastoma or other tumors commonly associated with RB mutation in humans (Clarke et al., 1992; Jacks, 1996; Jacks et al., 1992; Lee et al., 1992). Different from RB null or heterozygous mice, mice lacking either p107 or p130 develop normally and no tumor predisposition has been observed. The crosses between p130 and p107 (Cobrinik et al., 1996), RB and p107 (Lee et al., 1996), and RB and p130 (Mulligan and Jacks, 1998) have revealed both functional overlap and differences among the three family members. For example, all RB+/- p107-/- mice develop multifocal and bilateral retinal dysplasia whereas RB+/-p130-/-, p130+/- p107-/-, and p107+/- p130-/- mice don't (Mulligan and Jacks, 1998). Furthermore, by studying the differences between the acute

RB loss vs. germline RB loss, Sage *et al.* demonstrated that the compensation activities among RB family members are highly context dependent (Sage et al., 2003).

Similar to the original p53 knockout mice, mice lacking RB do not exhibit increased mammary tumor frequency (Clarke et al., 1992; Donehower et al., 1992; Jacks et al., 1992; Lee et al., 1992). In addition, both chimera and tissue grafting experiments indicate that loss of RB alone is not sufficient for mammary tumor formation (Maandag et al., 1994; Robinson et al., 2001). Perhaps, similar to the case of p53, genetic modifiers or permissive conditions are required for mammary tumor formation in RB -/- mice. It remains of interest to find out whether RB null mammary cells in a different genetic background such as in BALB/c will develop into mammary tumors. In other words, do modifying loci in BALB/c mice also increase mammary tumor susceptibility in RB null mammary glands.



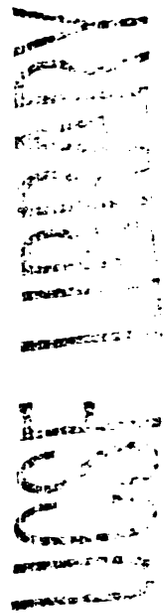
Another possibility is that p107 and/or p130 are sufficient to compensate for RB loss in mammary cells. To clearly address the specific contributions of RB family members, RB, p107, and p130, in suppressing mammary tumor initiation and progression, it is important to ablate all RB family members specifically in mammary cells. Since it is technically difficult to generate a triple knockout of essential genes that are required for mammary gland development, Simin *et al.* circumvented the difficulties by specifically expressing T_{121} in murine mammary glands under the control of the WAP promoter (Simin et al., 2004). T_{121} is the truncated variant of simian virus 40 (SV40) T antigen and is capable of inhibiting all three RB family members. Two deletions were made in T antigen to generate T_{121} variant. The 196bp N-terminal deletion abolishes small t antigen products and the *dl1137* deletion truncates T antigen so that it no longer binds to

and inhibits p53 (Simin et al., 2004). The expression of T_{121} in mammary glands leads to increased proliferation and apoptosis and leads to adenocarcinoma formation with a 16 month latency (Simin et al., 2004). This result demonstrates that RB family proteins are indeed important tumor suppressors in suppressing mammary tumor formation.

The importance of the RB pathway in suppressing mammary tumors is further demonstrated through studies of transgenic mice that exhibit deregulation of the RB pathway. Murine mammary gland models for upstream regulators of RB have also been generated and provide great insights into the RB-E2F pathway in mammary carcinogenesis. The RB tumor suppressor protein is phosphorylated and hence inhibited by cyclinD/cdk4/6 and cyclin E/cdk2. Over-expression of cyclin D, E, cdk2, or cdk4 as well as mutations in cdk2 that render it resistant to p16 inhibition have been detected in human breast cancers. In mouse models, all transgenic mice over-expressing cyclin D1 under MMTV promoter exhibit hyperplastic changes and ~66% mice develop multifocal mammary adenocarcinomas with papillary and cribriform elements and squamous differentiation with ~ 551 days of latency (Wang et al., 1994b). This result clearly demonstrates the tumor promoting functions of cyclin D. The oncogenic role of cyclin D1 in mammary gland tumor formation was further confirmed by Yu *et al.* By crossing cyclin D1 null mice with four different transgenic MMTV mice (*c-neu, v-Ha-ras, c-myc,* and *wnt-1*) that develop mammary tumors, they found that loss of cyclin D1 specifically prevented mammary tumors, but not other tumor types induced by either c-neu or v–Ha-Ras demonstrating the tumor-promoting function of cyclin D1 in mammary gland tumors induced by either c-neu or v–Ha-Ras (Yu et al., 2001a). Intriguingly, cyclin D1 is dispensable for c-myc and wnt-1 induced mammary tumors indicating the complexity of

the mammary tumorigenic process (Yu et al., 2001a). Subsequently, Gadd *et al.* found that cyclin D1 over-expression under the MMTV promoter abolishes the pulse of p16 expression that normal occurs and is essential for growth arrest during mammary gland involution (Gadd et al., 2001). Hence, it is possible that cyclin D1 over-expression may contribute to mammary tumorigenesis via inhibiting p16-induced arrest and promoting the expansion of a stem cell population (Gadd et al., 2001).

Similar to cyclin D1, over-expression of cyclin D 3 has also been seen in many cancers (Bartkova et al., 1996) and its expression in breast cancer correlates with high tumor grade (Wong et al., 2001). Transgenic mice over-expressing cyclin D3 under the MMTV promoter develop mammary carcinoma with a 73% frequency indicating the tumor promoting effect of cyclin D3 (Pirkmaier et al., 2003). Interestingly, cyclin D3 over-expressing mice develop squamous cell carcinomas of the mammary glands whereas cyclin D1 over-expressing mice primarily develop adenocarcinomas. These data suggest differences between the two cyclin proteins in cellular differentiation (Pirkmaier et al., 2003; Wang et al., 1994b).



Unlike cyclin D1 and D3, cyclin D2 levels are often reduced in human breast cancer and nearly 50% of human breast cancers exhibit cyclin D2 promoter silencing (Evron et al., 2001). Transgenic mice over-expressing MMTV-driven *cyclin D2* exhibit defects in alveologenesis and only a low frequency of tumor incidence (Kong et al., 2002). Interestingly, Kong *et al.* also found that over-expression of cyclin D2 leads to reduction in both hypo and hyperphosphorylated cyclin D1 (Kong et al., 2002). Considering the pivotal role of cyclin D1 in mammary tumor development, the inhibitory

effect of cyclin D2 on cyclin D1 may explain the frequent absence of cyclin D2 in human breast cancers.

Despite the fact that p16 loss is seen in approximately 30% of human breast cancers, mice lacking p16 do not exhibit increased incidence of mammary tumors (Krimpenfort et al., 2001; Sharpless et al., 2001). However, a recent study indicated that p16 inhibits cyclin D1 abundance in breast cancer cell lines (D'Amico et al., 2004). Perhaps p16's tumor suppressor role in mammary tumor development is exerted through inhibiting cyclin D1, a clear tumor-promoting gene in the mammary gland. Crossing a p16 null mouse with MMTV-driven *cyclin D1* transgenic mice will illuminate the role of p16, if any, in suppressing mammary tumors. Should the mice from such a cross exhibit increased tumor frequency and reduced tumor latency, p16 would indeed be an important tumor suppressor in mammary carcinogenesis. It also remains interesting to determine whether genetic modifiers or permissive conditions such as shortened telomeres cooperate with the loss of p16 to promote mammary tumor development. Consistent with p16 being a tumor suppressor in mammary carcinogenesis, mice with a knock-in of a $Cdk4^{R24C}$ germline mutation, which abolishes the ability of CDK4 to bind to and be inhibited by p16, develop various tumors including those of the mammary gland (Rane et al., 2002). Furthermore, Williams *et al.* reported that loss of $p16^{INK4a}$ co-operates with loss of caveolin-1 in promoting mammary hyperplasia (Williams et al., 2004).

Brca 1 and 2 in Mammary Gland Tumors

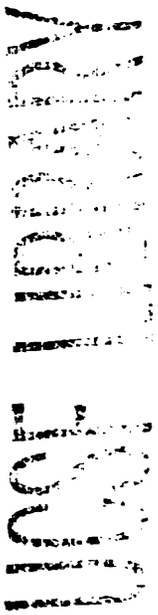
Several strains of mice with targeted deletions in various regions of *Brca 1* have been generated (Brodie and Deng, 2001; Deng, 2002). Mice with germline loss of Brca 1

function exhibit embryonic lethality due to deficiency in DNA damage repair, centrosomal and chromosomal abnormalities, cell cycle arrest and growth retardation, and increased apoptosis (Brodie and Deng, 2001; Deng, 2002). Early lethality for brca 1 -/- mice prior to mammary gland development obscures the role of brca 1 in suppressing mammary tumors. Brca 1 heterozygous mice, on the other hand, develop normally. And unlike humans who carry one mutant allele of *BRCA 1*, Brca 1 +/- mice do not exhibit increased mammary carcinoma frequency.

To reveal the specific function of Brca 1 in mammary tumors and generate a murine model of human BRCA1, a conditional knockout model was generated by crossing *Brca* 1-exon 11 floxed mice with either MMTV-*Cre* or WAP-*Cre* transgenic mice (Wagner et al., 1997; Xu et al., 1999). Mammary glands from the conditionally mutant animals demonstrated abnormal ductal and alveolar development and increased apoptosis (Xu et al., 1999). Approximately 23% of the mice developed mammary tumors at 11 month of age. Similar to their human counterparts, mammary tumor cells from the conditional knockouts displayed extensive genomic abnormalities and were highly aneuploid. A crossing between the *Brca 1* conditional knockout with p53 heterozygous mice accelerated mammary tumor development. The resulting *Brca1$^{co/co}$MMTV-Crep53*+/- mice exhibited increased tumor frequency and decreased latency (6-8 months for half the mice and 15 months for all) when compared to either *Brca1$^{co/co}$MMTV-Cre* or p53 +/- mice. Most tumors that arose in *Brca1$^{co/co}$MMTV-Crep53*+/- mice showed loss of the wild type *p53* allele (Xu et al., 1999). Taken together, these data demonstrate that mutations in *Brca1* and *Trp53* are co-operative in mammary gland tumorigenesis. Mutations in the "gatekeeper gene", p53, are likely to permit survival of cells with

mutation in the "caretaker gene", brca 1, and facilitate tumor development in Brca 1 mutants.

Like Brca 1 -/- mice, Brca 2 -/- mice also exhibit embryonic lethality (Suzuki et al., 1997). To circumvent the embryonic lethality seen in *Brca 2* knockout mice, conditional *Brca 2* knockout mice have also been generated using K14-*Cre* (Jonkers et al., 2001). Interestingly, *Brca2*$^{co/co}$*K14-Cre* mice did not develop epithelial tumors over a 30 months period. However, a reduction in p53 dosage was sufficient to facilitate mammary and skin tumor development within 10 months and all tumors that arose lost the wild-type p53 allele indicating the significance of p53 in monitoring tumors resulting from Brca 2 loss (Jonkers et al., 2001). The cooperation between the loss of Brca 1 or 2 and p53 deficiency in mammary tumorigenesis is not unique to mice. In human BRCA 1 or 2 breast cancers, p53 mutations are frequently detected.

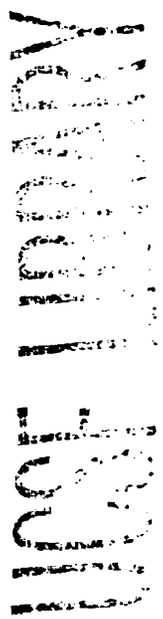


In Addition to GEM, Model Systems Employing Human Mammary Epithelial Cells are also Necessary to Comprehend Breast Cancer

Murine models have proven to be extremely useful systems to study breast cancer genetics due to the following reasons. First, a large repertoire of genetic tools is available for easy manipulation. Second, interbreeding provides ways to study co-operativity between different oncogenes and tumor suppressors and to identify genetic modifiers. Third, easy monitoring and follow-up permit studies of early stages of carcinoma as well as tumor progression.

However, mice are not human. About 50% of human breast cancers are ER-positive or responsive where as majority of mammary tumors in GEM are ER-negative,

or hormone independent (Nandi et al., 1995). Similarly, invasive breast cancers are frequently seen in humans, but most murine models of breast cancer do not exhibit metastatic lesions (Blackburn and Jerry, 2002). Mice containing mutations in most tumor suppressor genes are tumor prone, but they often do not phenocopy their human counterpart (Jacks, 1996; McClatchey and Jacks, 1998). Furthermore, murine cells may spontaneous immortalize whereas human primary cells including mammary epithelial cells are resistant to immortalization and often require multiple oncogenic actions (Hahn and Weinberg, 2002a; Hahn and Weinberg, 2002b). Transformation of human primary cells into tumorigenic variants requires a multistep process involving multiple genetic and/or epigenetic alterations, and each genetic and/or epigenetic alteration confers a proliferative advantage (Hahn and Weinberg, 2002a; Hahn and Weinberg, 2002b; Sherr and McCormick, 2002). The difference in immortalization ability may be in part explained by the difference in their telomere length. Human telomeres are about 10 kb in length whereas murine telomeres are up to 40-60 kb in length (Newbold, 1997) and adult murine somatic tissues express telomerase (Greenberg et al., 1998).



Given the numerous dissimilarities between mice and humans, it is essential to establish model systems that employ human mammary epithelial cells as well as murine models. Thus far, xenoplant models involving both heterotopic (kidney capsule or subcutenous) and othotopic (mammary fat pad) transplants and tissue culture models involving both 2D and 3D have been employed to study human breast carcinogenesis. Both heterotopic and othotopic xenoplant models using human mammary epithelial cells

are still in the early stage of development (Kuperwasser et al., 2004; Parmar et al., 2002) and are excitingly awaited to reach their full potential.

Culturing Human Mammary Epithelial Cells in 2D

Several research groups have independently developed techniques to isolate and culture human mammary epithelial cells (HMEC) from reduction mammoplasty tissues (Hammond et al., 1984; Kao et al., 1995; Pechoux et al., 1999; Stingl et al., 1998). These techniques differ in the ways of isolating and culturing the cells. Among them, the one developed by the Stampfer group at Lawrence Berkeley Laboratory was one of earliest and also most utilized (Hammond et al., 1984). Their culture conditions involve growing cells on plastic in serum free medium with only one undefined supplement, bovine pituitary gland extract. The ability to isolate HMEC from reduction mammoplasty of women, who have no indication nor predisposition to breast cancer, has made studying early steps of breast cancer possible since existing breast cancer cell lines would not be particularly useful to reveal early molecular events that initiate carcinogenesis. Furthermore, the Stampfer group was also successful in separately isolating and culturing isogenic fibroblast from the same reduction mammoplasty (http://www.lbl.gov/~mrgs/). This separation of HMEC and isogenic fibroblasts permits the independent examination of cell-type specific regulations. Studies from such independent analyses may provide insight into why breast cancers are largely derived from mammary epithelial cells and not from fibroblasts. In our laboratory, we employed the method developed by the Stampfer group because of its common acceptance, conveniently obtainable growth medium (from BioWhittaker), and easy manipulation and monitoring. In chapter 2, I will discuss our

attempts in identifying the early molecular alterations that may aid in the breast carcinogenic process using this model system.

One disadvantage of the Stampfer system is that HMEC are, unlike the *in vivo* situation, grown on plastic without the support of stromal cells. However, it can also be advantageous and valid to utilize a simplified system to begin to dissect complex biology. Without the knowledge gained through studies in simple model systems, the more complex model systems would only confuse us.

In an attempt to generate culturing systems that might mimic the *in vivo* situation, both bone marrow cells and mitomycin-treated 3T3 cells have been tried as possible stromal component added to the HMEC culture (Emerman et al., 1996; Ramirez et al., 2001; Wright and Shay, 2002). However, both models may not be appropriate representations since neither marrow cells nor 3T3 cells are part of the human mammary stroma. Mammary fibroblasts and/or myofibroblasts isolated from human breasts would be the better choice. Furthermore, mitomycin C-treated 3T3 cells may behave as activated fibroblasts such as those associated with carcinomas or radiation-activated stroma. Thinking of them as normal stromal components that support proper growth of mammary epithelial cells in culture may not be accurate. To demonstrate that irradiated mammary stroma behaves differently from the normal stroma and that alterations in the stromal response to radiation promote neoplastic progression, Barcellos-Hoff and Ravani transplanted initiated but weakly-tumorigenic mammary epithelial cells, COMMA-D, into either irradiated or non-irradiated mammary fat pads. They found that irradiated mammary stroma promotes tumorigenecity of un-irradiated mammary epithelial cells and increases the tumor incidence by 4 fold when compared to the sham-irradiated stroma

(Barcellos-Hoff and Ravani, 2000). Rapid remodeling of the stromal environment occurred upon radiation involving remodeling of extracellular matrix and activation of the latent tumor transforming growth factor β1 (TGF-β1) (Barcellos-Hoff, 2001). These alterations in stroma in response to irradiation may be responsible for the tumor promoting phenotypes. Perhaps in a similar way, Mitomycin-C, which also produces DNA damage, can cause alterations in the mouse 3T3 fibroblasts that promote neoplastic phenotypes.

Stromal-Epithelial Interactions in Breast Carcinogenesis

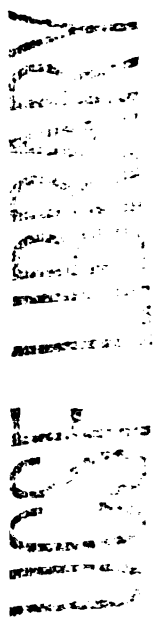
Even though it is mostly the mammary epithelial cells that exhibit increased proliferation, decreased apoptosis, and invasive ability, the mammary stroma, which consists of fibroblasts, adipocytes, pre-adipocytes, endothelial cells, inflammatory cells, and extra-cellular matrix (ECM), clearly participates in the mammary tumorigenic process by providing both suppressive (in the case of normal stroma), permissive, and instructive (in the case of altered mammary stroma) signals (Wiseman and Werb, 2002). The concept that stromal components of the breast can modulate the phenotypes of mammary epithelial cells is not new and should not be surprising. During mammary gland development, the proper cross-talk between the epithelial cells and the stroma is essential in ensuring proper mammary gland morphogenesis, patterning, and secretory functions of normal mammary gland (Fata et al., 2004; Wiseman and Werb, 2002). The disruption of this proper cross talk due to ECM remodeling and/or reactive fibroblasts, on the other hand, impairs mammary gland development and can also initiate and/or foster breast carcinogenesis (Bissell and Radisky, 2001; Fata et al., 2004; Radisky et al., 2002;

Shekhar et al., 2003; Wiseman and Werb, 2002).

Evidence demonstrating that alterations in the stromal components could initiate and/or promote tumorigenesis comes from the following experiments. First, the wound-healing response in stroma can influence tumor development (Dolberg et al., 1985; Sieweke and Bissell, 1994). Second, irradiation-induced stromal alterations promote tumorigenecity of weakly-tumorigenic mammary epithelial cells, COMMA-D (Barcellos-Hoff and Ravani, 2000). Third, targeted expression of the stromal protease MMP3/stromelysin-1, which degrades ECM, in mammary glands influences mammary tumor initiation and progression (Sternlicht et al., 1999). Fourth, signals from inflammatory cells, a component of the stroma, facilitate malignant transformation of mammary tumors (Lin et al., 2001). Fifth, chemical carcinogen-treated (*N*-nitrosomethylurea (NMU)) stroma promotes mammary gland tumorigenesis regardless whether or not the epithelial cells are treated with NMU, whereas control stroma does not even when the epithelial cells are exposed to NMU (Maffini et al., 2004). Sixth, targeted inactivation of the TGF-β type II receptor gene in mouse fibroblasts influences the tumorigenecity of adjacent epithelial cells in prostate and forestomach (Bhowmick et al., 2004). Careful examination of mammary phenotypes in these mice is anxiously awaited.

Studies from Allinen *et al.* also support the active effect of stroma in breast carcinogenesis. Allinen *et al.* separated the different cell types composing either normal or cancerous breast tissues and compared their gene expression profiles (Allinen et al., 2004). They found that substantial gene expression alterations were seen in all cell types isolated from the cancerous tissue, but the most consistent and dramatic gene expression alterations were found in the tumor myoepithelial cells and myofibroblasts. Furthermore,

many of the genes that exhibit altered expression encode secreted proteins and receptors. The authors demonstrated that two of the genes with altered expression in tumor myoepithelial cells and myofibroblast encode secreted CXCL12 and CXCL14 chemokines. Both chemokines are able to bind to their receptors on epithelial cells and enhance epithelial cell proliferation, migration, and invasion suggesting that they act in paracrine fashion to promote breast tumorigenesis (Allinen et al., 2004). In contrast, normal myoepithelial cells isolated from normal breast tissue, when co-cultured with various breast cancer cell lines in the presence or absence of additional fibroblasts, suppress cancerous cells' invasive ability (Jones et al., 2003). This invasion-suppressing effect of normal myoepithelial cells was achieved through down-regulation of metalloproteinase (MMP) expression in both cancer cells and fibroblasts via a paracrine fashion highlighting the importance of microenvironment on tumor development (Jones et al., 2003).



In breast cancer, emerging data have challenged the notion that the reason late-stage cancers are more invasive than the early-stage ones is the acquisition additional genetic mutations in the cancerous epithelial cells (Schedin and Elias, 2004). While it may be true that additional genetic mutations in the late-stage cancers can facilitate the invasion, an alternative explanation (though the two are not mutually exclusive) is that the tumor microenvironment plays a major role in determining whether a metastatic tumor cell will remain dormant or become invasive. This alternative hypothesis suggests that tumor cells possess metastatic potential early on, but only metastasize when permitted and/or promoted by the microenvironment. The evidence supporting, but far from proving, this alternative hypothesis comes from various microarray experiments

(Ma et al., 2003; Sorlie et al., 2001; Sorlie et al., 2003; van 't Veer et al., 2002; van de Vijver et al., 2002). Results from these experiments indicate that signature genes associated with metastasis can be found in at least a subpopulation of primary tumors and this metastatic potential in early-stage tumors is not limited to only a few rare cells. Similarly, the histologically pre-metastatic lesions can be partitioned into those with or those without metastatic potential. For those with metastatic potential, the gene expression profiles are similar between the pre-invasive and invasive lesions. Furthermore, early-stage premalignant lesions such as DCIS can exhibit similar gene expression profiles to that of invasive breast tumors. Finally, data from Allinen *et al.* indicate that the most consistent and dramatic gene expression changes occur in the stroma among normal breast tissue, DCIS, and invasive breast tumors, and at least two of the genes that are over-expressed in the myoepithelial cells and myofibroblast promote epithelial cell invasion (Allinen et al., 2004).

In summary, accumulating data indicate that the tumor microenvironment or stromal-epithelial interaction plays a key role in determining tumor initiation, progression, and invasion. Therefore, studies of human breast cancer development must take the stromal components into consideration in the future.

Culturing Human Mammary Epithelial Cells in 3D

Given the importance of stromal-epithelial interactions, it is essential to develop cell-based culture systems that better recapitulate the *in vivo* 3D organization and complexity of tissue than the 2D culture system does, and yet permits experimental manipulation and monitoring. Thus far, numerous 3D culture systems have been

developed ranging from spontaneous cell aggregation, 3D monotypic or multicellular spheroids in extracellular matrix, liquid overlay culture, gyratory rotation and spinner flask spheroid cultures, scaffold based cultures, to rotary cell culture systems (Kim et al., 2004b). Among them, the most commonly employed method is the 3D monotypic or multicellular spheroids in extracellular matrix. It was developed more than two decades ago and has been improved over time by Bissell and others (Bissell, 1981; Elsdale and Bard, 1972; Emerman and Pitelka, 1977; Ingber and Folkman, 1989; Schmeichel and Bissell, 2003). It is relatively inexpensive and best of all easily manipulated, monitored, and reproduced.

In 1982, Hall *et al.* showed that murine mammary epithelial cells developed acini-like structures with hollow lumen when plated in between two layers of ECM, collagen I (Hall et al., 1982). Similar acini-like structures were also observed for human mammary epithelial cells in collagen I (Howlett et al., 1995; Lelievre et al., 1998). However, the acini-like structures formed in collagen I exhibited abnormal polarity, disorganization, altered expression of integrin and other polarity markers, and do not differentiate properly to produce milk (Gudjonsson et al., 2002; Howlett et al., 1995; Streuli et al., 1991; Weaver et al., 2002). Addition of normal myoepithelial cells, but not myoepithelial cells isolated from breast cancers, to the collagen I matrix rescue the polarity defects and facilitate the formation of acini with center lumen (Gudjonsson et al., 2002) demonstrating the importance of myoepithelial cells in proper acini development and suggesting the valuable role of myoepithelial cells in maintaining proper polarity of normal breast tissues.

One of the functions of myoepithelial cells is to express laminins for generating basement membrane. To test whether it was the presence of proper basement membrane components after the addition of myoepithelial cells that promotes proper acini polarity, the authors reconstituted basement membrane components such as various laminins to the collagen I matrix. They found that among various laminins in human mammary basement membrane, laminin-1 could substitute for myoepithelial cells in rescuing the polarity defects (Gudjonsson et al., 2002). Consistently, when embedded in the laminin-rich matrigels derived from murine Engelbreth-Holm-Swarm tumors (Kleinman et al., 1986), human mammary epithelial cells also develop polarized spherical structures similar to that of normal mammary acini, express proper integrins, and differentiate to produce milk as normal mammary acini do (Bissell et al., 2003; Howlett et al., 1995; Petersen et al., 1992; Streuli et al., 1991; Weaver et al., 1997). In addition to ECM, Huss and Kratz showed that adipocytes may also facilitate proper acini development (Huss and Kratz, 2001). Together, these data demonstrate that human mammary epithelial cells respond to the spatially restricted biochemical cues provided by the normal myoepithelial cells and/or extracellular matrix for the accurate presentation of multi-cellular phenotypes.

A single epithelial cell, at least in the case of MCF-10A (a non-transformed mammary epithelial cell line), initially proliferates and subsequently enters growth arrest to form a spherical structure when suspended in matrigel (Debnath et al., 2002). The cells in the center of the spherical structure that do not adhere to the basement membrane die of apoptosis to create the center lumen and develop acini-like structure (Blatchford et al., 1999; Debnath et al., 2002; Muthuswamy et al., 2001). This phenomenon is

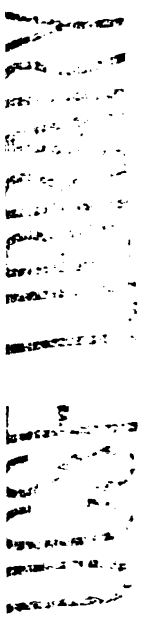
physiologically relevant to involution, which is in part due to the transient disruption of the basement membrane by metalloproteinases at the end of lactation. This data is consistent with the notion that normal ECM provides survival cues for epithelial cells that adhere (Frisch and Francis, 1994). Whereas cells that are detached from ECM die of apoptosis (anoikis) (Frisch and Francis, 1994). Boudreau *et al.* later showed that inappropriate ECM, such as collagen I, only temporarily delayed the anoikis. Only relevant ECM, such as basement membrane, maintained long-term survival (Boudreau et al., 1995). Now, the interesting question is what spatially restricted biochemical and biological cues provided by the appropriate ECM suppress apoptosis of epithelial cells that adhere to it.

To answer this question, Reginato *et al.* showed that detachment from matrigel down-regulates EGF receptor (EGFR) and up-regulates a pro-apoptotic gene, Bim, among several BH3-only proteins (Reginato et al., 2003). This up-regulation of Bim upon detachment is required for the apoptotic process since down-regulation of Bim via RNA interference (RNAi) inhibits anoikis. Furthermore, Bim up-regulation requires β1-integrin disengagement and down-regulation of EGFR, both of which occur upon detachment from the matrigel or upon the addition of β1-integrin blocking antibody (Reginato et al., 2003). Hence, the appropriate β1-integrin signaling mediated via proper stromal-epithelial cell interactions can act dominantly and epigenetically to influence epithelial cell phenotypes. The β1-integrin engagement between proper stromal and epithelial cells may also help to explain why only relevant ECM such as matrigel can sustain long-term survival since mammary epithelial cells may express different integrins when suspended in different ECM.

Interestingly, apoptosis mediated by the loss of β1-integrin engagement is not necessary for lumen formation since Bcl-2 over-expression, which blocks apoptosis, does not block lumen formation (Debnath et al., 2002; Reginato et al., 2003). Under this situation, autophagy is sufficient to create central lumen in the absence of apoptotic process (Debnath et al., 2002). Similarly, over-expression of oncogenes, such as cyclin D1 or HPV E7 that enhance proliferation but do not affect apoptosis, does not block lumen formation (Debnath et al., 2002). These data indicate that over-proliferation or inhibition of apoptosis alone is not sufficient to block lumen formation and that acini structure is resistant to isolated oncogenic insult. However, consistent with multi-step carcinogenesis, autophagy alone is no longer able to generate center lumen and mammary epithelial cells develop into distorted, depolarized, and filled 3D structures when both increased proliferation and inhibition of apoptosis are achieved through over-expression of oncogenes such as ErbB2 or HPV E7 together with bcl-2 (Debnath et al., 2002; Muthuswamy et al., 2001). Under these situations, apoptosis is necessary for lumen formation.

Human mammary epithelial cells isolated from breast tumors, on the other hand, exhibit deregulated proliferation and abnormal 3D structure, do not deposit endogenous basement membrane, and do not respond to the signaling cues provided by the ECM when suspended in laminin rich matrigels (Petersen et al., 1992; Roskelley and Bissell, 2002). These phenotypes of cancerous mammary epithelial cells in matrigel are often associated with the activation of β1-integrin at the expense of α6β4 integrin required for normal polarity establishment (Nievers et al., 1999) and the up-regulation of EGFR (Wang et al., 1998; Weaver et al., 1997). Inhibition of β1-integrin and EGFR signaling

by blocking antibodies revert the malignant phenotype of a breast cancer cell line to polarized and growth arrested acini-like structures (Wang et al., 1998; Weaver et al., 1997). Furthermore, blocking β1-integrin signaling down-regulates both endogenous β1-integrins and the levels of EGFR whereas inhibition of EGFR reduces the level of β1-integrin and normalizes β1-integrin signaling. Significantly, this cross-modulation between EGFR and β1-integrin only occurs in a 3D context (Liu et al., 2004; Wang et al., 1998; Weaver et al., 1997). Taken together, these data strengthen the argument that appropriate 3D culture system is essential in dissecting the complex biological signaling networks during mammary gland development and carcinogenesis.



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Chapter 2

Histologically Normal Human Mammary Epithelia with Silenced p16INK4a Over-express COX-2, Promoting a Premalignant Program

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Summary

Breast tissue from healthy women contains variant mammary epithelial cells (vHMEC) exhibiting p16INK4a promoter hypermethylation both in vivo and in vitro. When continuously cultured, vHMEC acquire telomeric dysfunction and produce the types of chromosomal abnormalities seen in pre-malignant lesions of cancer. We find that late passage vHMEC express elevated prostaglandin cyclo-oxygenase 2 (COX-2), which contributes to increased prostaglandin synthesis, angiogenic activity, and invasive ability. These data demonstrate the existence of human mammary epithelial cells with the potential to acquire multiple genomic alterations and phenotypes associated with malignant cells. Moreover, COX-2 over-expression coincides with focal areas of p16INK4A hypermethylation in vivo creating ideal candidates as precursors to breast cancer. These putative precursors can be selectively eliminated upon exposure to COX-2 inhibitors in vitro.

Significance

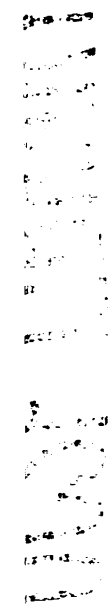
Our studies provide molecular markers for identifying perhaps the earliest lesions of human breast cancer (i.e., pre-malignant mammary epithelial cells) and a molecular mechanism for their generation. Furthermore, our data indicate that these vHMEC with malignant phenotypes can be selectively eliminated, thus preventing their further progression. Finally, our study suggests that NSAIDs can directly target epithelial cells for cancer prevention, in addition to its effect in targeting stromal and inflammatory cells. These data point to a possible molecular mechanism through which nonsteroidal anti-inflammatory drugs (NSAIDs) reduce breast cancer risk.

Introduction

Breast tissue from healthy women, when examined *in vitro*, contains a subpopulation of variant mammary epithelial cells (vHMEC, post-selection, M0 or post-senescent HMEC) that have silenced $p16^{INK4a}$ through hypermethylation of promoter sequences (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998). These cells exhibit several properties that distinguish them from the majority of mammary epithelial cells that proliferate from a tissue explant (HMEC or pre-selection HMEC) including the silencing of $p16^{INK4a}$ gene expression through promoter hypermethylation and the stabilization of p53 (Romanov et al., 2001). In culture, vHMEC proliferate an additional 30 – 50 generations beyond the time that the HMEC population activates a proliferative arrest, before reaching a second population growth plateau, termed agonescence (Romanov et al., 2001). Remarkably, nearly 100% of vHMEC approaching agonescence exhibit telomeric dysfunction and acquire chromosomal defects, including aneuploidy, telomeric associations, and various other classes of structural abnormalities similar to those seen in the earliest lesions of breast cancer (Holst et al., 2003; Romanov et al., 2001; Tlsty et al., 2001). In a recent study, we found that a subpopulation of histologically normal human mammary tissues *in vivo* also contain cells with hypermethylated $p16^{INK4a}$ promoter sequences. These cells were found in discrete foci in a substantial fraction of women with no indication or predisposition to breast cancer (Holst et al., 2003). We hypothesize that this subpopulation of vHMEC, which exists *in vivo,* has potential for progression to pre-malignant, and ultimately malignant, lesions of the breast. Therefore, characterization of these cells *in vitro* may provide molecular markers

for identifying pre-malignant lesions of breast cancer *in vivo*, as well as, critical targets for therapeutic or preventive intervention.

In this study, we find that cyclo-oxygenase 2 (COX-2), a gene that is often over-expressed in many human cancers and contributes to colon cancer initiation and progression in murine models (Oshima et al., 1996), is up-regulated in $p16^{INK4a}$ hypermethylated mammary epithelia *in vitro*. This up-regulation of COX-2 contributes to increased prostaglandin synthesis, increased endothelial cell invasion and invasive ability in vHMEC, and thus provides growth advantages to already genomically unstable cells. Ominously, we found that cells with $p16^{INK4a}$ promoter hypermethylation and co-incident intense COX-2 expression exist in histologically normal human tissues *in vivo*, creating ideal candidates for breast cancer precursors.



Results

Human Mammary Epithelial Cells in Agonescence Express High Levels of COX-2

To identify novel properties that distinguish vHMEC from HMEC, we compared the gene expression profiles of both cell populations as they progressed through increasing population doublings using microarrays. Primary explants from reduction mammoplasties from women ranging in age from 16 to 57 years were cultured as described previously to generate the growth curves for HMEC and vHMEC (Figure 2-1A). For this study, RNAs from cells at different passages in culture were compared to identify markers that change as the cells approach agonescence (Figure 2-1A). Among the genes that were up-regulated in vHMEC at later passage or agonescence was COX-2 (an ~6 fold induction between late and early vHMEC, Figure 2-1A, inset). This gene was of immediate interest since several lines of evidence implicate COX-2 protein as an important modulator of tumor progression (Howe et al., 2001). We verified that the up-regulation of COX-2 mRNA was indeed accompanied by an increase in protein expression via Western analysis (Figure 2-1B) and immunocytochemistry (Figure 2-1C). In these analyses, COX-2 expression was undetectable in the early HMEC population but increased significantly as vHMEC populations approached and entered agonescence (Figure 2-1B and C). Similar COX-2 up-regulation in vHMEC was observed in samples derived from three additional individuals (Figure 2-1C and microarray data not shown). In a fifth case, low levels of COX-2 expression were observed in HMEC (RM 9) and vHMEC from the same mammoplasty demonstrated similar COX-2 expression to that of the other vHMEC populations.

We further investigated the timing, heterogeneity and level of COX-2 over-expression in individual vHMEC cells. Immunocytochemistry analysis demonstrated that, while COX-2 was expressed at a high level in the majority of vHMEC at late passage, the protein was expressed at a similarly high level but in far fewer cells in vHMEC populations at early passage. Thus, the majority of the increase in COX-2 expression was the result of the induction of an increased number of cells exhibiting high expression (Figure 2-1C, right column) rather than a general increase in all cells. These data indicate that the loss of $p16^{INK4a}$ protein *per se* is not responsible for the induction of COX-2 since all early vHMEC cells in the population exhibit $p16^{INK4a}$ promoter hypermethylation, but only a few cells over-express COX-2. Furthermore, expression of exogenous $p16^{INK4a}$ protein in vHMEC over-expressing COX-2 does not suppress COX-2 over-expression (data not shown). Taken together, these data suggest that the lack of p16 activity may be permissive for a subsequent event, which then leads to the induction of COX-2 over-expression. Once this subsequent event occurs, the consequences cannot be suppressed by $p16^{INK4a}$ expression.

High Levels of COX-2 Expression can be Induced by DNA Damage Associated with Telomeric Dysfunction

An event that occurs subsequent to the loss of $p16^{INK4a}$ protein activity and coincides with the increase in COX-2 over-expression is the increase in the number of cells exhibiting telomeric dysfunction (Figure 2-2A). Numerous studies have described how telomeric dysfunction can activate DNA damage response pathways (de Lange, 2002; Nautiyal et al., 2002). Therefore, we investigated the possibility that DNA damage signals such as those generated by telomeric dysfunction could induce COX-2. To

experimentally induce a DNA damage response that mimics telomeric dysfunction, we exposed early passage vHMEC (with negligible COX-2 expression) to adriamycin, which was previously reported to cause telomeric dysfunction and result in telomere-related chromosomal abnormalities in breast tumor cells (Elmore et al., 2002). We found that early passage vHMEC exhibited a strong induction of COX-2 expression upon exposure to 1μM adriamycin (Figure 2-2B). Additionally, we exposed early passage vHMEC to inhibitors for topoisomerases I and II (campothecin and etoposide, respectively), two agents that also generate DNA breaks similar to those produced by telomeric dysfunction (i.e. dicentric chromosomal breakage). Under both of these conditions, COX-2 was induced in a dose dependent manner (Figure 2-2B and C, and data not shown). A recent study demonstrated that DNA damage, induced by mitomycin C, also up-regulated COX-2 (Han et al., 2002). Taken together, our data support the interpretation that DNA damage similar to that produced by telomeric dysfunction, an event that occurs in vHMEC subsequent to $p16^{INK4a}$ promoter hypermethylation, can contribute to the induction of COX-2 over-expression.

High Levels of COX-2 Expression in Agonescent vHMEC Confers Malignant Phenotypes

While the downstream effects of over-expressing COX-2 in tumor cells are well established, the phenotypes that accompany COX-2 over-expression in vHMEC are unknown. Comparison of vHMEC expressing a high level of COX-2 to those with a negligible level revealed significant differences in the expression of phenotypes hypothesized to be critical to malignancy (e.g. control of prostaglandin synthesis, angiogenesis, invasion, and apoptosis). vHMEC expressing a high level of COX-2

demonstrated a dramatic increase in the induction of prostaglandin E_2 (PGE_2), a direct product of COX-2 activity, as well as three other downstream products of COX-2: 6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$, and thromboxane B_2 (TXB_2), when compared to cells expressing a negligible level of COX-2 (Figure 2-3A and B, and data not shown) (RM16 and 48). Furthermore, vHMEC expressing a high level of COX-2 stimulated a significant increase in the invasion of human umbilical vein endothelial cells (HUVEC) across a collagen layer when compared to HMEC expressing a negligible level of COX-2 in an assay for inducing angiogenic ability (Figure 2-3C, black bars) in three vHMEC population tested (RM15, RM16, and 48). Finally, vHMEC expressing a high level of COX-2 induced increased invasion of mammary epithelial cells across a Boyden chamber barrier when compared to HMEC expressing a negligible level of COX-2 (Figure 2-3D, black bars) in three vHMEC population tested (RM 15, RM16, and 48). Hence, both vHMEC and tumor cells, each over-expressing COX-2, exhibit similar phenotypes that contribute to malignant potential. Interestingly, conditioned media from the COX-2-overexpressing vHMEC did not demonstrate an ability to induce either HUVEC or mammary epithelial cell invasion across a collagen layer (Figure 2-3C and 3D, white bars). These data suggest that the downstream signal(s) of COX-2 that mediate both angiogenic and invasive activity is transient or short-lived. Thus, constant COX-2 activity is required for the invasive phenotype induced by vHMEC.

To determine if the specific expression of COX-2 is necessary and/or sufficient for the exhibition of these malignant phenotypes, we manipulated COX-2 levels and documented the causal relationship between the two events. To remove elevated COX-2 expression from late passage vHMEC we used both a chemical inhibitor and a COX-2

anti-sense construct. We exposed the late passage vHMEC to a COX-2 inhibitor, NS-398, for 24 hours and measured HUVEC invasion 12 hours later. We found that the originally observed HUVEC invasion (Figure 2-3C) was substantially reduced upon exposure to NS-398 (Figure 2-4A). We next introduced a COX-2 anti-sense construct under the control of tetracycline promoter into late vHMEC over-expressing COX-2 (RM16: PD 38 and 48: PD 48). Four days after the addition of doxycycline, COX-2 protein levels were reduced in vHMEC containing a COX-2 anti-sense construct (Figure 2-4E, top panel). Furthermore, this reduction in COX-2 protein level was accompanied by a reduced level of excreted PGE_2 (Figure 2-4B). When these cells (RM16 and 48) were assayed for their ability to stimulate HUVEC invasion or movement of vHMEC across transwell barrier, both phenotypes were substantially attenuated, dropping to the same levels observed after exposure to NS-398 (Figure 2-4C and D). Expression of an anti-sense RNA may trigger an interferon response as a consequence of dsRNA formation and an altered pattern of protein expression. To exclude the possibility that the activation of an interferon response caused the reduction in the phenotypes observed above, we induced an interferon response in our vHMEC cells (RM15: PD 25 and RM 16: PD 30) by exposing them to human interferon β. We found that the addition of interferon β (5-100 unit/ml) for 2-6 days was sufficient to up-regulate the expression of a cell surface protein, HLA-1, as previously reported (Satoh et al., 1995) (Supplementary Figure 2-S1). However, despite the activation of interferon response in these vHMECs, neither HUVEC invasion nor movement of vHMEC across transwell barrier was reduced (Supplementary Figure 2-S2). Furthermore, the HLA-1 protein level was similar in cells expressing either a COX-2 anti-sense or the vector control (Supplementary Figure 2-S3)

and the growth kinetics (or population doublings) (Supplementary Figure 2-S4) for vHMEC containing either a COX-2 anti-sense or a control construct remain the same after the induction of COX-2 anti-sense expression for a period of time, including the 4 days during which all the relevant experiments were done. Together our data indicate that COX-2 plays a causal role in the induction of these phenotypes observed in late vHMEC.

To investigate the sufficiency of COX-2 in promoting the phenotypes observed, we introduced exogenous COX-2 into early vHMEC (RM15: PD 7, RM16: PD 10, and 48: PD 10) that exhibited a low level of endogenous COX-2 expression (Figure 2-4E, bottom panel). This increase in COX-2 protein level was accompanied with an increased level of excreted PGE_2 (Figure 2-4F) and we found an increase in HUVEC and vHMEC invasion phenotypes (Figure 2-4G and H) in all three vHMEC populations tested (RM15, RM16, and 48, only data for RM15 is shown). Interestingly, early vHMEC expressing exogenous COX-2 often demonstrated only a modest increase in the HUVEC invasion phenotypes when compared to late passage vHMEC over-expressing endogenous COX-2 (Figure 2-3C) despite the fact that their COX-protein levels were similar (data not shown). This data suggest that early vHMEC, but not late vHMEC, may still maintain the ability to modulate COX-2 mediated phenotypes. Nevertheless, our data suggest that COX-2 over-expression alone is able to increase the HUVEC and vHMEC invasion phenotype. Together, our data demonstrate that COX-2 over-expression in late vHMEC is causal for the phenotypes that promote malignant potential.

Foci of Histologically Normal Human Mammary Epithelia with p16INK4a Promoter Methylation Over-express COX-2 in Vivo

Our previous work identified focal aggregates of mammary epithelial cells that contained methylated $p16^{INK4a}$ promoter sequences in a subgroup of reduction mammoplasties obtained from healthy women (Holst et al., 2003). As reported here, *in vitro* cultured vHMEC with methylated $p16^{INK4a}$ promoter sequences have the ability not only to accumulate chromosomal abnormalities, but also to induce malignant phenotypes by up-regulating COX-2. These cells would be ideal candidates for precursors to pre-malignant lesions of breast cancer, an observation that provoked our search for these cells *in vivo*. As reported earlier, cells within histological sections containing the methylated $p16^{INK4a}$ promoter sequences retained normal tissue architecture as confirmed by hemotoxylin and eosin (H&E) staining of adjacent serial sections (Holst et al., 2003). Strikingly, COX-2 staining of the adjacent serial sections demonstrated that only samples with foci containing ductal or lobular epithelial cells with $p16^{INK4a}$ promoter hypermethylation (yellow areas, Figure 2-5) exhibited intense COX-2 staining (red demarcations, Figure 2-5, samples 9698 and 9624). This co-localization of COX-2 protein staining and $p16^{INK4a}$ promoter hypermethylation within the same cells is presented in a novel mapping technique (Figure 2-5 and Holst et al., 2003) and examples of COX-2 staining are shown. In some places, the areas of intense COX-2 staining are seen beyond the individual ducts and lobules that contain luminal mammary epithelial cells with hypermethylation of $p16^{INK4a}$ promoter sequences in the surrounding epithelial structures. This is possibly due to the diffusion of COX-2 products (*e.g.*, prostaglandins) to the immediately adjacent areas, which up-regulate COX-2 expression (Tjandrawinata and Hughes-Fulford, 1997). Alternatively, this may be due to an underestimate of the

number of ducts and lobules that contain cells with hypermethylated $p16^{INK4a}$ promoter sequences. The mammary tissue that expresses high COX-2 levels may have hypermethylation of adjacent CpG islands that are beyond the region of the $p16^{INK4a}$ promoter sequences we investigated via methylation-specific-PCR. In one sample, 10811, COX-2 staining was less intense than that observed in the other two samples with $p16^{INK4a}$ silencing (Figure 2-5, sample 10811). This may reflect a cell population that has not yet fully sustained the events subsequent to $p16^{INK4a}$ promoter hypermethylation that allow for full COX-2 induction or, alternatively, a cell population that has processed the events so that they no longer induce COX-2. Low to undetectable expression of COX-2 was observed in the majority of the tissue from the seven samples that were devoid of $p16^{INK4a}$ promoter hypermethylation. Expression of COX-2 was largely devoid in surrounding stromal areas independent of $p16^{INK4a}$ promoter hypermethylation status in the epithelial cells. These data demonstrate that foci of vHMEC *in vivo* exhibit elevated COX-2 expression.

vHMEC with High Levels of COX-2 Expression Apoptose when Exposed to a COX-2 Inhibitor both in Two-Dimensional Culture and in Three-Dimensional Mammospheres

These newly described attributes of vHMEC have important clinical implications. Since COX-2 is known to inhibit apoptosis, inhibition of COX-2 functions may reinstate the apoptotic response and curtail the expansion of cells over-expressing COX-2. To this end, we exposed HMEC and vHMEC to various concentrations of NS-398, a specific COX-2 inhibitor (Liu et al., 1998), for 5 days and assayed its effect on viability. As shown in Figure 2-6A, B and C, vHMEC that expressed high levels of COX-2 protein

exhibited a significant decline in viability and activation of apoptosis as demonstrated by TUNEL analysis (Figure 2-6B). Upon exposure of the cells to NS-398 for 5 days, the majority of late passage vHMEC died while, under identical conditions, the majority of the (non-COX-2 over-expressing) HMEC remained viable (Figure 2-6A, B and C). This analysis was performed on four isogenic sets of HMEC and vHMEC with similar results (RM15, RM16, 48, and 240).

It has often been observed that therapeutic agents that are effective in two-dimensional assays may be ineffective when the cells are grown in three-dimensional structures (Hoffman, 1993). To better assess the potential of COX-2 inhibitors *in vivo*, we suspended HMEC and vHMEC (RM16 and 240) in reconstituted basement membrane where they formed three-dimensional structures (mammospheres), and exposed these mammospheres to NS-398. Our results demonstrate that even when in three-dimensional structures, the COX-2 inhibitor effectively killed the COX-2 over-expressing vHMEC while having a negligible effect on HMEC (Figure 2-6D).

Discussion

In Vivo Foci of Histologically Normal Mammary Epithelial Cells Have Silenced a Tumor Suppressor and Activated a Tumor Promoting Program

Excitingly, our studies identify an *in vivo* population of mammary epithelial cells in disease-free women that have the ability to silence the tumor suppressor gene, $p16^{INK4a}$, through promoter hypermethylation and over-express the tumor promoting gene, COX-2. Our studies also identify an *in vitro* population of variant mammary epithelial cells from disease-free women that, by loss of certain cell cycle checkpoint controls, have the ability to accumulate chromosomal abnormalities and express phenotypes that are critical to malignant progression. Without inactivation of pathways that prevent proliferation past a barrier, vHMEC could not proliferate to the point of telomeric crisis and undergo the sustained subsequent induction of COX-2. Thus, the initial checkpoint control defect, in part due to the epigenetic modulation of $p16^{INK4a}$, plays a crucial role in funneling cells into malignant pathways. The striking co-localization of COX-2 expression and $p16^{INK4a}$ promoter hypermethylation within the same cells both *in vivo* and *in vitro* emphasizes the relevance of our *in vitro* studies in dissecting the early pathways leading to carcinogenesis.

Importantly, these studies also identify therapeutic targets that would allow for the elimination of these variant cells if they prove relevant to human disease. If variant cells were involved in disease progression, one would expect to find them in at least a fraction of premalignant lesions. Our recent finding of COX-2 over-expression in the majority of ductal carcinoma in situ (DCIS) lesions lends credence to this hypothesis (Shim et al., 2003). Even more intriguing was the finding that the histologically normal epithelial cells

surrounding DCIS lesions, which were either positive or negative for COX-2 over-expression, also demonstrated increased expression of COX-2. These observations suggest that cells with over-expression of COX-2 may provide a fertile field for the emergence of premalignant lesions and, as discussed below, could be excellent targets for preventive therapy.

Several of the "Hallmarks of Cancer" are Co-Coordinately Controlled by COX-2 Expression

The co-localization of intense COX-2 staining in cells with hypermethylated $p16^{INK4a}$ sequences has important implications for the initiation and progression of malignancy in breast tissue. COX-2 expression has been shown to be accompanied by phenotypes that are critically relevant to cancer development (Howe et al., 2001), several of which have been designated as "hallmarks of cancer" (Hanahan and Weinberg, 2000). COX-2 catalyzes the production of prostaglandins (PG_s) including prostaglandin E_2 (PDE_2), which stimulates mammary epithelial cell proliferation in the presence of EGF (Bandyopadhyay et al., 1987), and estrogen synthesis by activating aromatase (Harris et al., 1999). COX-2 induced PDE_2 also mediates immunosuppression by inhibiting T and B-lymphocytes and cytokine production (Howe et al., 2001; Huang et al., 1998). Furthermore, the over-expression of COX-2 in tumor cells regulates angiogenesis, invasion and apoptosis by promoting production of pro-angiogenic, pro-invasive and anti-apoptotic factors (Gately, 2000; Tsujii and DuBois, 1995). Therefore, COX-2 expression in human mammary cells may provide sustained growth signals to mammary epithelial cells, protection from immune surveillance, and expression of malignant phenotypes. Our data (Figures 2-3 and 2-6) suggest that vHMEC containing

hypermethylated *p16*^{INK4a} promoter sequences and COX-2 over-expression exhibit malignant phenotypes such as increased angiogenesis and invasion and decreased apoptosis. Thus, they possess several characteristics identified as the "hallmarks of cancer" (Hanahan and Weinberg, 2000) and may represent a *bona fide* pre-malignant population. One characteristic these cells do not exhibit is unlimited replicative potential. In these cells the loss of p16^{INK4a} allows an extended proliferative potential, but not immortalization. Thus, we hypothesize that the over-expression of COX-2 enhances their malignant potential, priming them for malignant transformation, but additional alterations may be required for full transformation.

DNA Damage and Genomic Instability Leads to the Induction of COX-2 and the Activation of Stromal and Epithelial Phenotypes Crucial in Malignant Progression

Genomic instability plays a pivotal role in initiating and promoting the expression of malignant phenotypes on several levels. In human mammary epithelial cells, the hypermethylation of *p16*^{INK4a} promoter sequence abrogates selected cell cycle checkpoint controls (Meyer et al., 1999) and allows the HMEC to bypass an *in vitro* growth barrier whose origin is currently unknown (Brenner et al., 1998; Romanov et al., 2001) (Figure 2-7). This loss of p16 protein relieves cyclin D1/cyclin dependent kinase 4/6-control during G1/S transition and allows the subsequent unrestrained phosphorylation of pRB by cdk4/6 (T.D.T, unpublished results). In the absence of these restraints and the presence of adequate nutrients and mitogenic factors, the vHMEC have an extended replicative potential (Brenner et al., 1998; Romanov et al., 2001). As vHMEC further proliferate, they continue to erode telomeric sequences until they experience telomeric dysfunction and initiate a cascade of genomic instability (Romanov et al., 2001).

Telomere dysfunction not only provides genetic mutations required for cancer initiation (Maser and DePinho, 2002) but also instigates an apoptotic response (Karlseder et al., 1999). Studies in this report suggest that vHMEC with telomere dysfunction may also oppose these death signals by inducing COX-2, an anti-apoptotic regulator, and thus allow the further proliferation of otherwise unviable cells. Concomitantly, these cells acquire malignant phenotypes that act within the epithelial cell as well as in the surrounding stroma (i.e., increased angiogenic response). The COX-2-induced inhibition of apoptosis and immune surveillance, along with the acquisition of invasive abilities, generate a population of epithelial cells that are poised for malignant progression. Furthermore, the COX-2-induced remodeling of the stroma along with the induction of angiogenesis supports this malignant program. Thus, we postulate that the two events of genomic instability, epigenetic modulation of $p16^{INK4a}$ and chromosomal breaks generated by telomeric dysfunction, provide the initiating and promoting forces that drive the acquisition of a premalignant program (Figure 2-7). Subsequent induction of COX-2 (and other gene alterations) provide growth advantages to these otherwise compromised cells. While we do not suggest that all cancers arise in this manner, we do suggest that the critical events in this process (loss of cell cycle checkpoint control, telomere dysfunction, DNA damage, and sustained induction of COX-2) provide a mechanistic framework for malignant transformation.

NSAIDS Target Over-expression of COX-2 in Mammary Epithelia in Addition to Stroma Acting Both Early and Late in Malignant Progression

The first convincing evidence linking COX-2 to tumor initiation and progression came from studies in APC-deficient, COX-2-null mice (Oshima et al., 1996). The lack of

COX-2 (either through genetic or pharmacological manipulation) reduces the incidence of intestinal adenomas and tumor size (Alshafie et al., 2000; Harris et al., 2000; Nakatsugi et al., 2000; Robertson et al., 1998). While the removal of COX-2 function suppresses tumor initiation and progression, the over-expression of COX-2 exacerbates the process in murine models. Oshima et al provide genetic evidence that the induction of COX-2 is an early, rate-limiting step for adenoma formation (Oshima et al., 1996). Furthermore, tissue-specific over-expression of COX-2 from a mouse mammary tumor virus (MMTV) promoter was sufficient to cause mammary tumors in multiparous mice (Liu et al., 2001). These results in murine models strongly suggest that COX-2 is an important modulator of both tumor initiation and progression.

In humans, COX-2 also plays a dual role in carcinogenesis. The over-expression of COX-2 is often observed in multiple human tumors including breast, colon, pancreas, and prostate (Soslow et al., 2000; Tucker et al., 1999). Additionally, and consistent with our view that COX-2 may play a role in early steps of carcinogenesis, epidemiological data suggests regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin, which inhibits COX-2, reduces both sporadic and familial colon cancer incidence (Gupta and Dubois, 2001; Thun et al., 1991). Other studies point to a similar NSAID effect for breast and pancreatic cancer incidence (Friedman and Ury, 1980; Harris et al., 1996; Schreinemachers and Everson, 1994; Sharpe et al., 2000). A recent large prospective cohort study involving over 80,000 postmenopausal women indicated that there was a 21% and 28% reduction in the risk of breast cancer for women who took NSAIDs at least twice a week for at least 5 to 10 years when compared to women who reported no or minimal NSAIDs usage (Harris et al., 2003). These data, in conjunction

with our observations of histologically normal human tissue in this report and studies from murine models, suggest that COX-2 functions both early and late in tumor progression and highlight COX-2 as a potential target for preventive therapy.

Summary

In summary, we demonstrate intense expression of COX-2 protein co-localized with $p16^{INK4a}$ promoter hypermethylation in human mammary epithelial cells both *in vitro* and *in vivo*. COX-2 expression in these cells parallels the acquisition of telomeric dysfunction and can be induced by DNA damage. Further, COX-2 over-expression together with $p16^{INK4a}$ promoter hypermethylation generates critical phenotypes that are believed to be instrumental in the progression to malignancy. Our data also indicate that NSAIDs can directly target epithelial cells for cancer prevention, in addition to its effect in targeting stromal and inflammatory cells. Finally, the identification of differentially expressed genes in vHMEC not only allows for the identification of early lesions of breast cancer but also suggests targets for the selective removal of these putative precursor cells from the body.

Experimental procedures

Cells and cell culture. Isolation and culture of HMEC in modified MCDB 170 (MEGM, BioWhittaker, USA) has been described (Hammond et al., 1984). We studied HMEC/vHMEC from reduction mammoplasty specimens from five individuals: HMEC/vHMEC 48, HMEC/vHMEC 240 (kindly provided by Martha Stampfer) and HMEC/vHMEC RM9, RM15, and RM16 (cells derived in our laboratory). Population doublings (PD) 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.

Microarray Experiment. Total RNA was isolated from HMEC (individual 48) using Qiagen Rneasy midi kit (Qiagen, Valencia, CA). Filled red circles in Figure 2-1A indicate points in the growth curve when mRNAs were isolated. The microarray experiments were performed at the National Institute of Environmental Health Sciences Microarray Center (Research Triangle Park, NC). Detailed protocols for microarray procedures are available at http://dir.niehs.nih.gov/microarray.

Immunohistochemistry. Five-micron sections were cut from routinely fixed paraffin embedded tissue blocks and mounted on SuperFrost plus microscope slides (Fisher Scientific, Pittsburgh, PA). Specimens were stepwise deparaffinized in xylene and rehydrated in descending alcohols. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in methanol for 10 min. Sections were microwaved for antigen retrieval for 10 min in a 10mM citrate buffer, pH 6.0. Sections were rinsed in PBS, incubated for 30 min in blocking buffer (10% horse serum, and 1% bovine serum albumin [BSA] in PBS) and immunostaining was performed using a COX-2 specific anti-human mouse monoclonal antibody (Cayman Chemical, No. 160112, Ann Arbor, MI) diluted 1:200 in 1% BSA in PBS overnight at 4°C. Control sections received human COX-2 control peptide (40 μg/ml; Cayman Chemical) along with the COX-2 primary

antibody (incubated for 1 hour prior to application). Sections were rinsed in 0.05% Tween-20 in PBS followed by incubation in a biotinylated IgG anti-mouse secondary (Vector Laboratories, No. BA-2000, Burlingame, CA) made in horse, diluted 1:200 in 1% BSA in PBS. Slides were rinsed in PBS and incubated in avidin-biotin horseradish peroxidase complex (Vector Laboratories, No. PK-6100, Vectastain Elite ABC kit) at a 1:100 dilution in 1% BSA in PBS for 30 min. Specimens were rinsed in 0.05% Tween-20 in PBS and incubated with 3,3′-diaminobenzadine (DAB) chromogenic substrate (Sigma Chemical, No. D-5905, St. Louis, MO) for 4 min. Sections were counterstained in hematoxylin, stepwise dehydrated through graded alcohols, and cleared in xylene before mounting using permount.

Western analysis. 30 μg of protein from total cell extracts was fractionated in gradient (4%-20%) polyacrylamide gels (FMC) and transferred to Hybond-P (Amersham) membrane. Lysates were sequentially exposed to rabbit polyclonal anti-human COX-2 antibody (Oxford Research Biochemical and/or Cayman Chemical Co.) followed by horse-radish peroxidase (HRP)-conjugated goat-anti-rabbit antibody (Gibco).
Flow analysis. Cells were fixed with 1% PFA and permeablized with 0.3% triton X-100. Subsequent flow analysis was performed using FITC conjugated anti-COX-2 antibody (Cayman Chemical, No. 160113).

Measurement of angiogenesis and invasion. Various HMEC and vHMEC were plated onto 12 well plates at a density that would reach 80% confluency three days later. HUVEC (from ATCC) or HMEC were plated onto inserted chambers at a density of $3x10^4$/chamber. The number of cells moving across a collagen layer was determined by trypan blue staining and adjusted to the number of HMEC or vHMEC in each 12 well plate. Fold of induction in invaded HUVEC or HMEC was determined when compared to HMEC, which was then set to be one. Each experiment was done in triplicate. For experiments involving cells with vector only, COX-2 anti-sense construct, or COX-2

over-expression construct, fold changes were determined when compared to vector control cells, which was set to be one. Each experiment was done six times.

PGE_2 measurement. PGE_2 was determined using Prostaglandin E_2–Monoclonal Enzyme Immunoassay kit (Cayman Chemical). Each experiments was carried out in triplicate according to the manufacturer's protocol.

Expression of COX-2 sense and anti-sense constructs. COX-2 sense construct was packaged in phoenix A cells for viral propagation. Viral supernatant was diluted 1:1 with MEGM media and then added to the early vHMEC twice consecutively. vHMEC infected with retroviruses were selected and maintained in 1μg/ml puromycin. The tetracycline-on gene expression plasmid pUHD.2neo expressing the COX-2 anti-sense was transfected into late vHMECs using lipofectamine 2000 (Invitrogene). Stable clones of vHMEC containing COX-2 anti-sense construct were selected and maintained in 70 μg/ml of Geneticin (GibcoBRL). To induce the expression of COX-2 anti-sense, 2 μg/ml doxycycline (Clontech) was added to the media prior to the experiments.

NS-398 inhibition. NS-398 (Caymen Chemical Company, Ann Arbor, Michigan) was dissolved in DMSO to generate 10 mM and 100 mM stock solutions. All final concentrations of NS-398 used for the experiments were diluted in the same amount of DMSO and made fresh for each usage. For two-dimensional culture, vHMEC were trypsinized and plated at a density of $2x10^4$ cells per well in a 6 well plate for exposure of cells to 0, 10, and 25 μM and $5x10^4$ per well for cells exposed to 50 μM NS-398. HMEC were treated similarly except $3.5x10^4$ cells per well were plated for exposure to 50 μM NS-398. NS-398 was applied to cells one day after initial seeding and every other day thereafter via a media change. Cells were harvested on the sixth day and the numbers of live and total cells for each experiment were determined using the trypan blue exclusion assay. Percent survival was calculated as the number of live cells divided by the number

of total cells in 0 μM NS-398, and adjusted according to the initial number of cells seeded. The relative percent survival for cells in 0 μM NS-398 was set to be 100%. The relative percent survival for cells in 10, 25, and 50 μM were calculated as the ratio between the percent of survival at various NS-398 concentrations and the percent survival at 0 μM NS-398. This experiment was done in triplicate for each of four sets of HMEC/vHMEC pairs. For 3D culture, HMEC/vHMEC were trypsinized and suspended in Matrigel (BD Bioscience, Bedford, MA) at a density o f $5x10^4$ cells/ 300μl. Cells were allowed to form 3D structures for 10 days followed by exposure to 0, 50, 100 μM NS-398. Five days after applying NS-398, cells were stained with propidium iodide (PI) without fixation and imaged using IP lab image software.

TUNEL Assay. TUNEL experiments were performed according to the manufacturer's protocol from Clontech.

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Figure 2 - 1

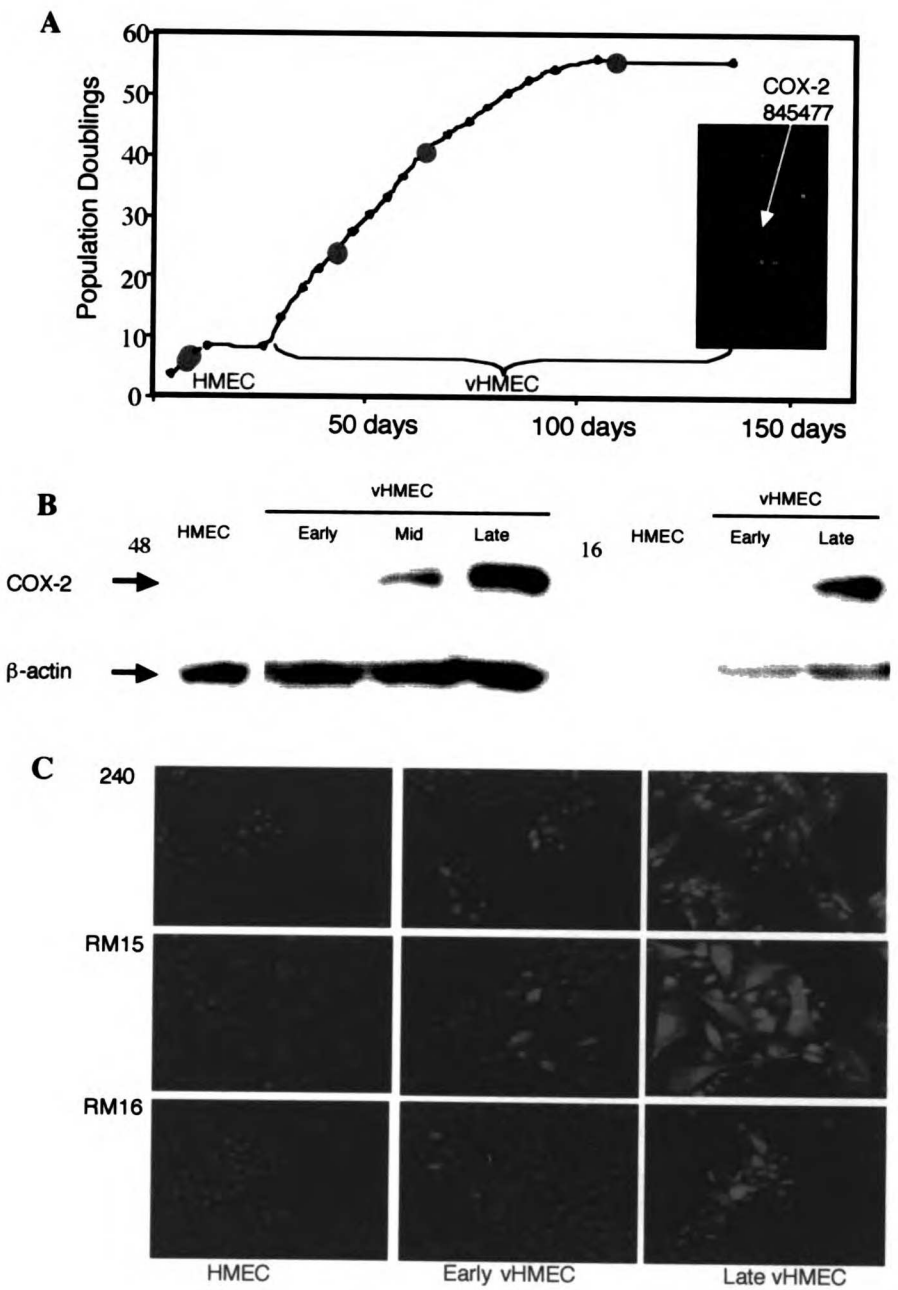

A
Population Doublings
60
50
40
30
20
10
0
COX-2
845477
HMEC
vHMEC
50 days
100 days
150 days
B
48
HMEC
vHMEC
Early
Mid
Late
16
HMEC
vHMEC
Early
Late
COX-2
β-actin
C
240
RM15
RM16
HMEC
Early vHMEC
Late vHMEC

Figure 2-1. COX-2 expression is elevated in vHMEC *in vitro*. A, Representative HMEC/vHMEC 48 growth curve. Filled red circles indicate population doublings (PD) at which mRNA was isolated for microarray experiment. Insert, an example of microarray data showing the up-regulation of COX-2 (arrow and red color). B, Expression of human COX-2 protein quantity assessed by Western blot analysis. Lysates from HMEC/vHMEC 48 were loaded: HMEC (PD 3), early passage vHMEC (PD 21), mid passage vHMEC (PD 34), late passage vHMEC (PD 44). Lysates from HMEC/vHMEC RM16 were loaded: HMEC (PD 4), early passage vHMEC (PD 18), late passage vHMEC (PD 38). C, Immunocytochemistry for COX-2 expression in HMEC, early, and late passage vHMEC 240 (PD 3, PD 25, and PD 46), RM15 (PD 3, PD 19, and PD 48), and RM16 (PD 3, PD 13, and PD 44) respectively.

Figure 2 – 2

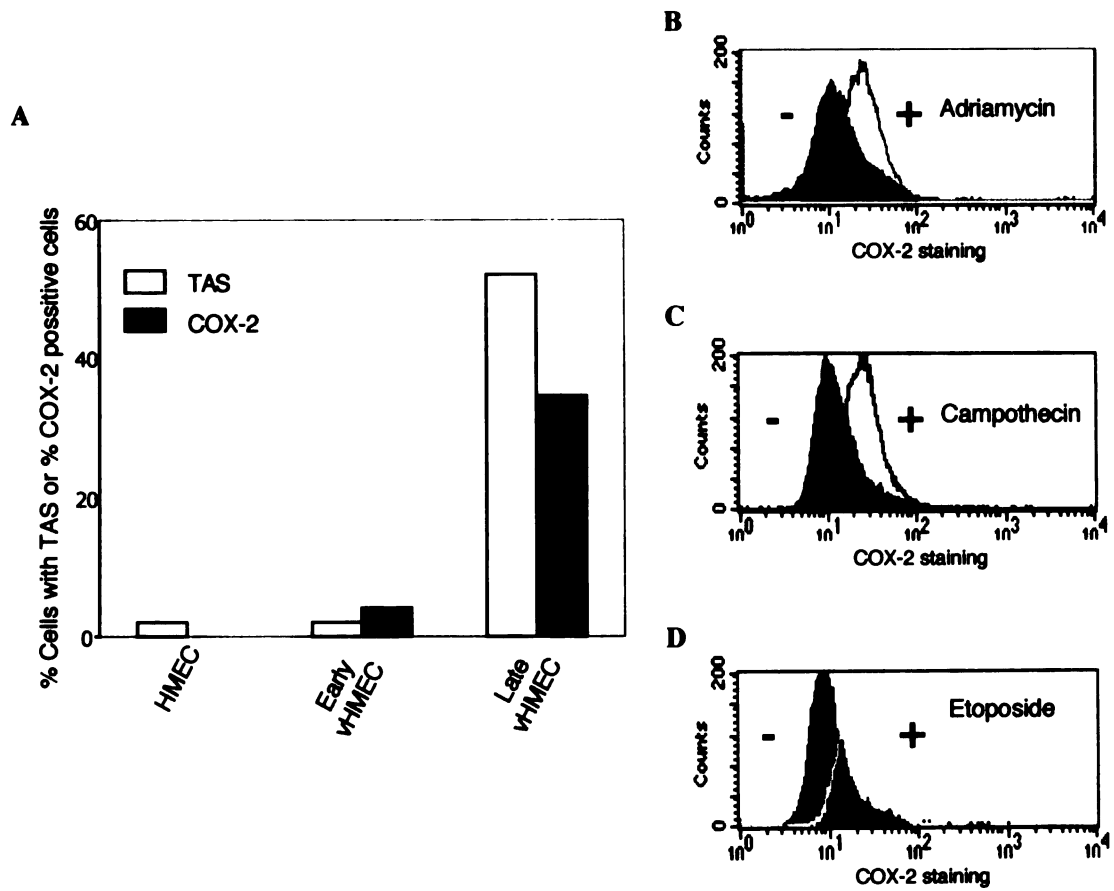

A
% Cells with TAS or % COX-2 possitive cells
60
40
20
0
TAS
COX-2
HMEC
Early vHMEC
Late vHMEC
B
Counts
200
0
-
+
Adriamycin
COX-2 staining
C
Campothecin
D
Etoposide

Figure 2-2. DNA damage induces COX-2 expression. A, Percent COX-2 positive vHMEC (48) increases as percent vHMEC exhibiting telomere association (TAS) increases. Percent COX-2 positive cells were determined using flow cytometry. TAS was scored as dicentric telomeres after G-banding karyotyping. B-D, Flow analysis indicates DNA damaging agents, adriamycin (1 μM), campothecin (2.5 μM), and etoposide (2.5 μM) induce COX-2 expression. Early vHMEC (RM16: PD 13 or PD 18) were incubated with adriamycin for 2 hours, and with campothecin or etoposide for 6 hours, and analyzed 24 hours later.

Figure 2 – 3

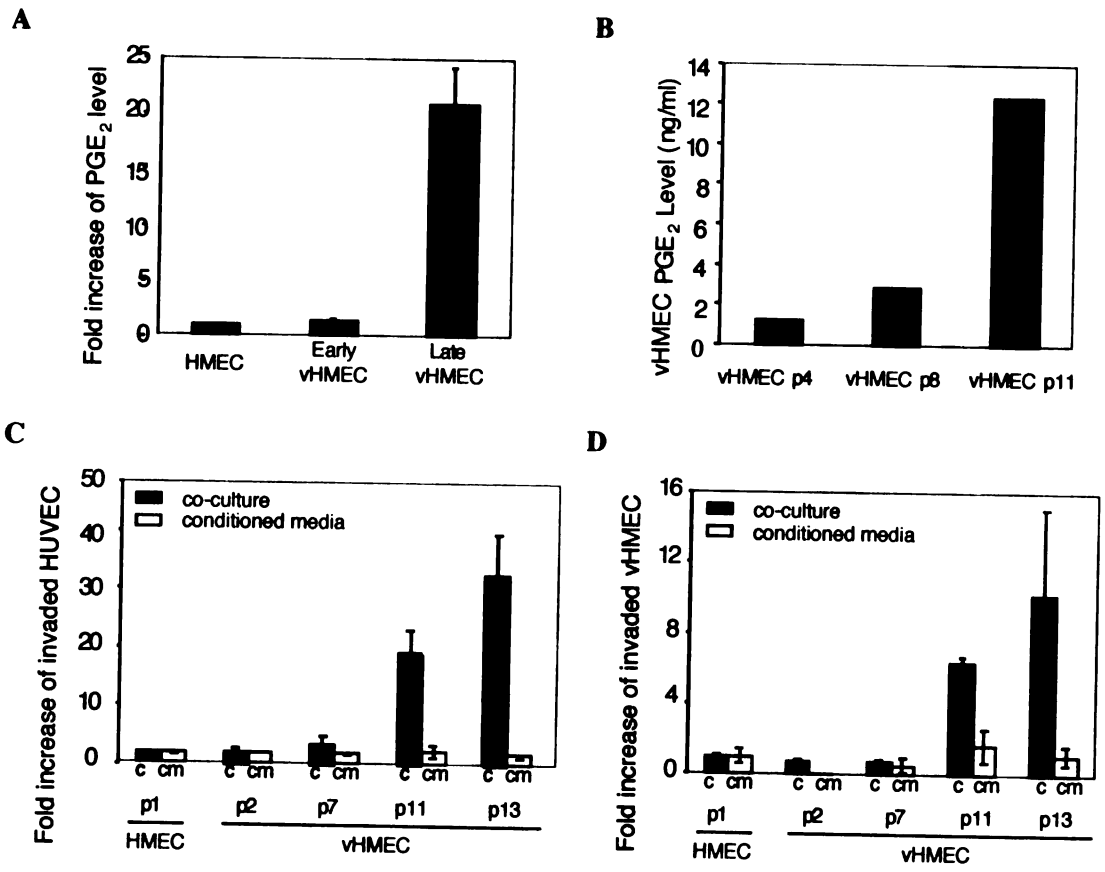

A
Fold increase of PGE2 level
25
20
15
10
5
0
HMEC
Early vHMEC
Late vHMEC
B
vHMEC PGE2 Level (ng/ml)
14
12
10
8
6
4
2
0
vHMEC p4
vHMEC p8
vHMEC p11
C
Fold increase of invaded HUVEC
50
40
30
20
10
0
co-culture
conditioned media
c cm
p1
HMEC
p2
p7
p11
p13
vHMEC
D
Fold increase of invaded vHMEC
16
12
8
4
0
co-culture
conditioned media
c cm
p1
HMEC
p2
p7
p11
p13
vHMEC

Figure 2-3. Late passage, COX-2-over-expressing vHMEC exhibit increased PGE_2 production, angiogenic potential, and invasive ability. A, Fold induction in PGE_2 levels. PGE_2 levels were determined using ELISA essay (Cayman Chemical) and normalized to the number of cells (RM 16) harvested. The level of PGE_2 for HMEC was set to be one. Fold of PGE_2 induction for vHMEC was determined as the ratio of PGE_2 levels for vHMEC vs. HMEC (mean ± S.E.M). RM 16 HMEC, early, and late vHMEC were at PD 5, PD 20, and PD 43 respectively. B, PGE_2 concentration in media (determined using mass spectrometry) increases as vHMEC (RM 16 p4 or PD 20, p8 or PD 34, and p11 or PD 43) approach agonescence. C, Late passage vHMEC (RM 16), but not conditioned media from late passage vHMEC, exhibit increased ability to summon HUVEC. "c" (black bar) means the experiment was done with HMEC or vHMEC. "cm" (white bar) means conditioned media was used for the experiment. D, Late passage vHMEC (RM 16), but not conditioned media from late passage vHMEC, exhibits increased ability to induce mammary epithelial cell invasion across a collagen layer. RM16 HMEC/vHMEC p1, p2, p7, p11, p13 were at PD 2, PD 13, PD 30, PD 44 and PD 49 respectively. All experiments in C-D were done in triplicate and all data were normalized to the number of cells harvested. Data obtained for HMEC or conditioned media from HMEC within each experiment were set to be one, and fold induction for vHMEC were determined as the ratio of vHMEC vs. HMEC (mean ± S.E.M).

Figure 2 - 4

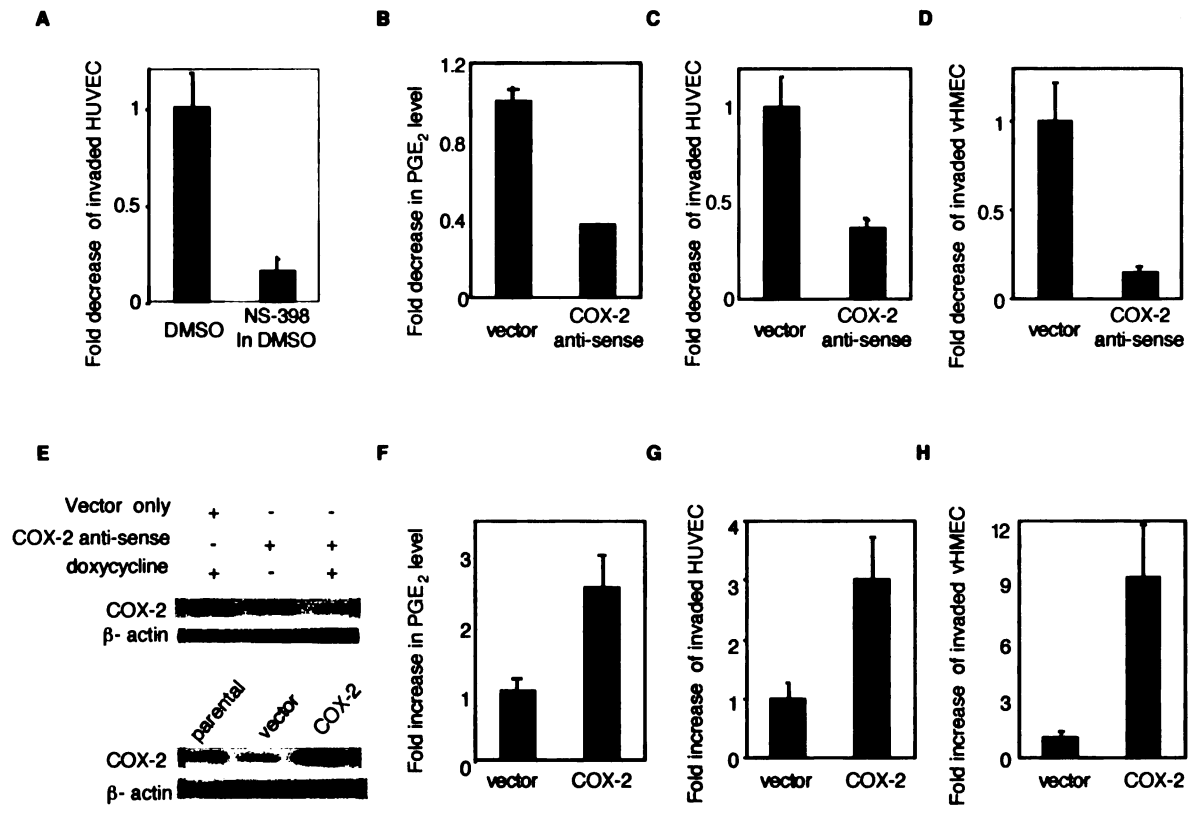

A
Fold decrease of invaded HUVEC
1
0.5
0
DMSO
NS-398 In DMSO
B
Fold decrease in PGE2 level
1.2
0.8
0.4
0
vector
COX-2 anti-sense
C
Fold decrease of invaded HUVEC
1
0.5
0
vector
COX-2 anti-sense
D
Fold decrease of invaded vHMEC
1
0.5
0
vector
COX-2 anti-sense
E
Vector only + - -
COX-2 anti-sense - + +
doxycycline + - +
COX-2
β- actin
parental
vector
COX-2
COX-2
β- actin
F
Fold increase in PGE2 level
3
2
1
0
vector
COX-2
G
Fold increase of invaded HUVEC
4
3
2
1
0
vector
COX-2
H
Fold increase of invaded vHMEC
12
9
6
3
0
vector
COX-2

Figure 2-4. COX-2 contributes to the phenotypes observed in late vHMEC. A, COX-2 specific inhibitor, NS-398 (25 μM), reduces the angiogenic activity in late vHMEC (RM16: PD 44). The experiment was carried out as above except that DMSO or NS-398 in DMSO was added to the media 24 hours before the assay. Data obtained for DMSO control was set to be one, and fold change for vHMEC in NS-398 was determined as the ratio of vHMEC/NS-398 vs. vHMEC/DMSO. Note: Addition of NS-398 (25 μM) did not kill vHMEC within the 36 hour period exposure time. B - D, Expression of COX-2 anti-sense RNA reduces PGE_2 level, and reduced HUVEC and vHMEC invasion phenotypes, respectively, in late vHMEC (RM16: PD 44). E, Top panel: Western analysis for COX-2 protein level in late vHMEC with either vector control or COX-2 anti-sense with or without the addition of Doxycycline (RM16: PD 44). Bottom panel: Western analysis for COX-2 protein level in early vHMEC (RM15: PD 7) with either vector or COX-2 over-expression construct. F - H, Over-expression of COX-2 leads to increased PGE_2 level, and increased HUVEC and vHMEC invasion phenotypes, respectively, in early vHMEC (RM15: PD 7). Similar results were observed with other RMs.

Figure 2 - 5

A

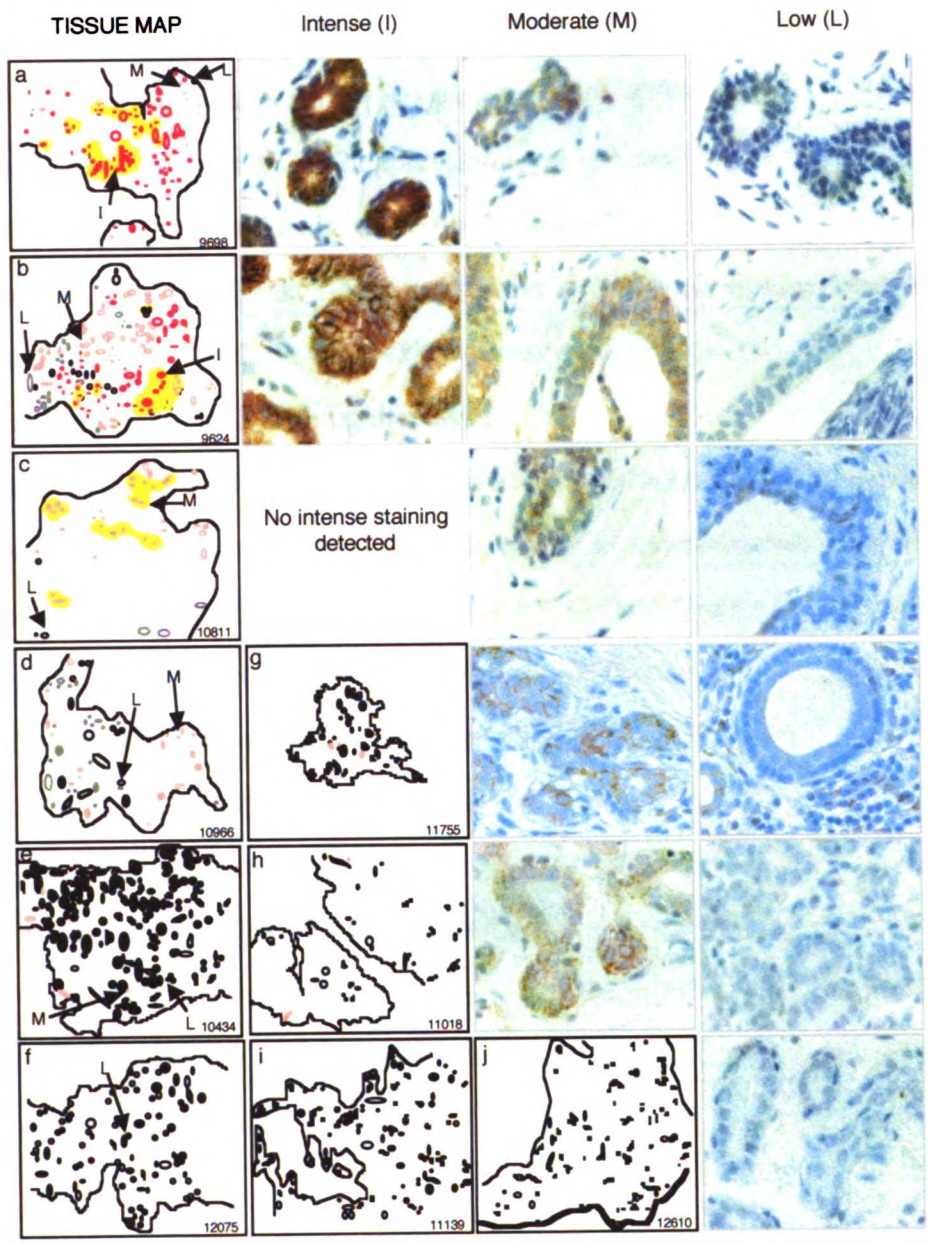

TISSUE MAP
Intense (I)
Moderate (M)
Low (L)
a
M
L
I
9698
b
M
L
I
9624
c
M
L
10811
No intense staining detected
d
M
L
10966
g
11755
e
M
L
10434
h
11018
f
L
12075
i
11139
j
12610

Figure 2-5. Intense COX-2 staining coincides with focal areas of $p16^{INK4a}$ promoter hypermethylation in histologically normal tissue *in vivo*. a-j. We generated a gridded map of epithelial cell clusters by examining the H&E section at 40x magnification. All samples were determined to be histologically normal. Ductal clusters are illustrated as open ovals, lobular clusters as filled ovals, and foci of $p16^{INK4a}$ promoter hypermethylation as yellow areas in a, b, c (samples 9698, 9624 and 10811, respectively). In the adjacent serial sections, COX-2 expression level was assessed by immunohistochemical-staining, and then scored as intense (red, >50% cells staining at highest intensity), moderate (pink, 1-50% cells staining with heterogeneous low to moderate intensity) or low (black, 0% - <5% cells staining with low intensity). The examples of moderate COX-2 staining displayed in rows 4 and 5 are examples of the most intense staining observed in samples d and e. The few epithelial cell clusters that could not be assessed due to processing problems are gray. Regions of adipose tissue are not indicated for simplicity. Samples (a) 9624 (b) 9698 and (c) 10811 each contain areas of $p16^{INK4a}$ promoter hypermethylation as previously identified by in situ hybridization detection of methylation-specific PCR. Samples (d-j) 10966, 10434, 12075, 11755, 11018, 11139, and 12610 respectively, were previously determined to be devoid of foci with $p16^{INK4a}$ promoter hypermethylation. The arrows indicate representative areas of intense (I), moderate (M) or low (L) staining for selected samples within that row. Note that the seven samples that were devoid of $p16^{INK4a}$ promoter hypermethylation did not contain any areas of intense COX-2 staining and in most instances contained either few or no areas of moderate staining.

Figure 2 - 6

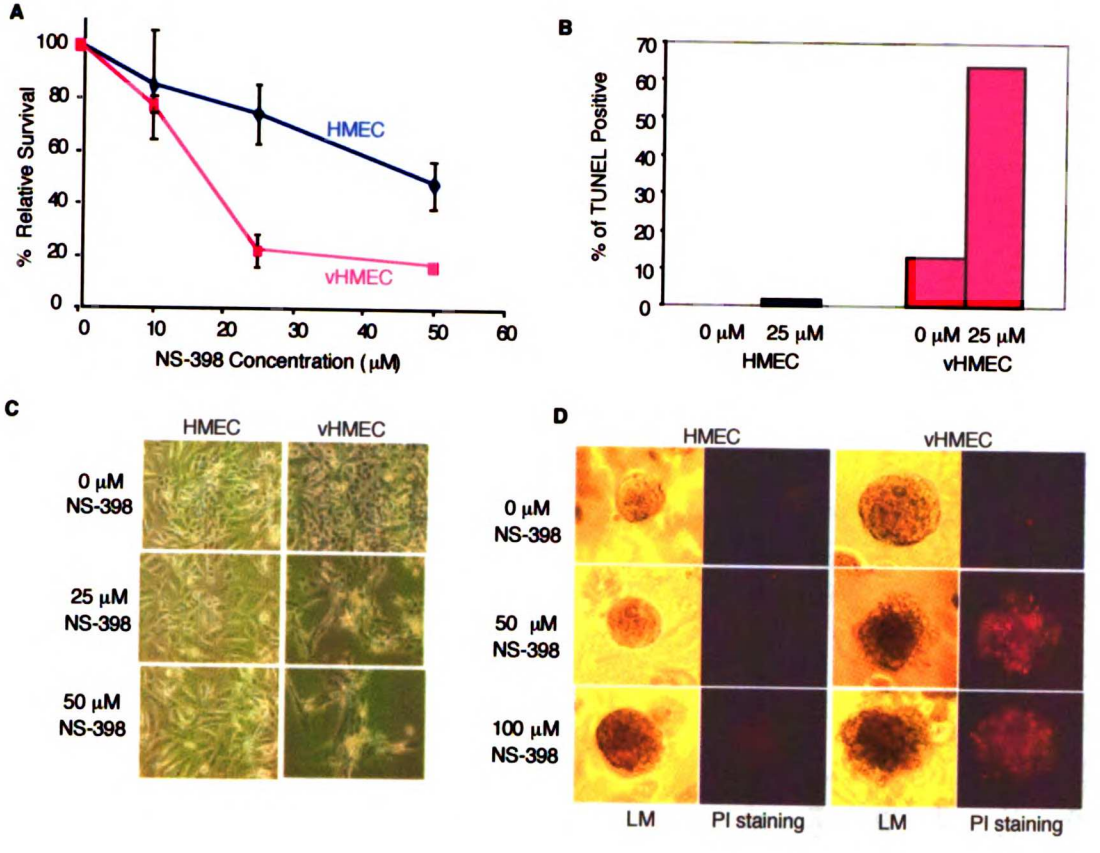

A
% Relative Survival
NS-398 Concentration (μM)
HMEC
vHMEC
B
% of TUNEL Positive
0 μM 25 μM
HMEC
0 μM 25 μM
vHMEC
C
HMEC
vHMEC
0 μM NS-398
25 μM NS-398
50 μM NS-398
D
HMEC
vHMEC
0 μM NS-398
50 μM NS-398
100 μM NS-398
LM
PI staining
LM
PI staining

Figure 2-6. COX-2 inhibitor reduces cell survival of COX-2-expressing vHMEC. A, Percent survival of HMEC and vHMEC (RM16: PD 4 and PD 34) in the presence of various concentration of NS-398 after 5 days of incubation (mean ± S.E.M). B, TUNEL experiment demonstrating the percentage of apoptotic cells after exposure to 25 μM NS-398 (RM16: PD 4 and PD 37). C, Phase image of RM16 HMEC/vHMEC (PD 4 and PD 34) after incubation with 0, 25 and 50 μM NS-398 for 5 days. D, Light microscopy (LM) and Propidium iodine (PI) staining of HMEC and vHMEC (sample 240: PD 4 and PD 44) mammospheres in 3D-culture after incubation with 0, 50 and 100 μM NS-398 for 5 days. Light microscopy illustrates the disrupted architecture of vHMEC mammospheres after incubation with NS-398. PI staining (red color) indicates cell death within vHMEC mammospheres after incubation with NS-398.

Figure 2 - 7

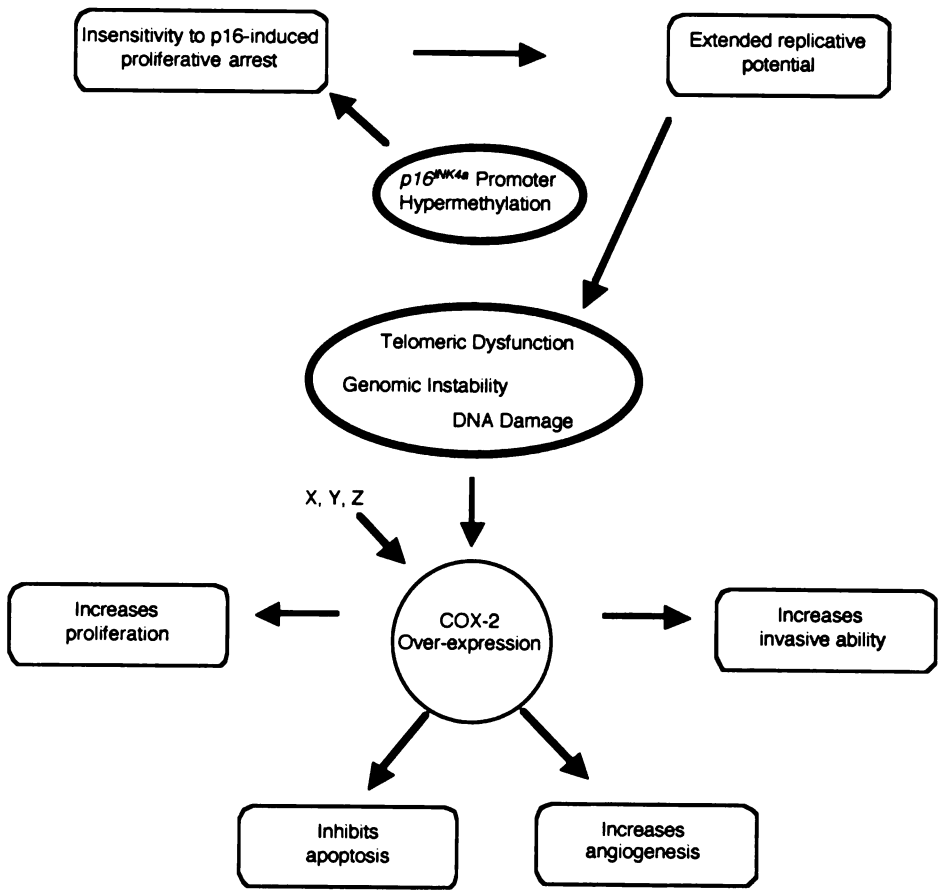
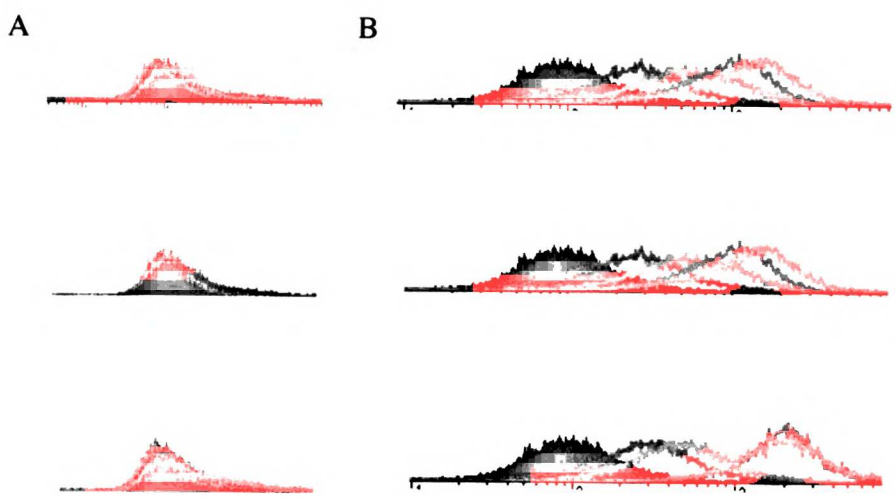

Insensitivity to p16-induced proliferative arrest
Extended replicative potential
p16INK4a Promoter Hypermethylation
Telomeric Dysfunction
Genomic Instability
DNA Damage
X, Y, Z
Increases proliferation
COX-2 Over-expression
Increases invasive ability
Inhibits apoptosis
Increases angiogenesis

Figure 2-7. Genomic instability potentiates premalignant programs. *p16*^{INK4a} promoter sequence hypermethylation allows cells to proliferate until acquiring telomeric dysfunction. Genomic instability generated by chromosomal breakage associated with telomeric dysfunction is pivotal in activating multiple phenotypes associated with cancer thru the up-regulation of a single gene, COX-2. COX-2 can also be induced via other pathways indicated in the figure as X, Y, Z.

Supplementary Figure 2-S 1

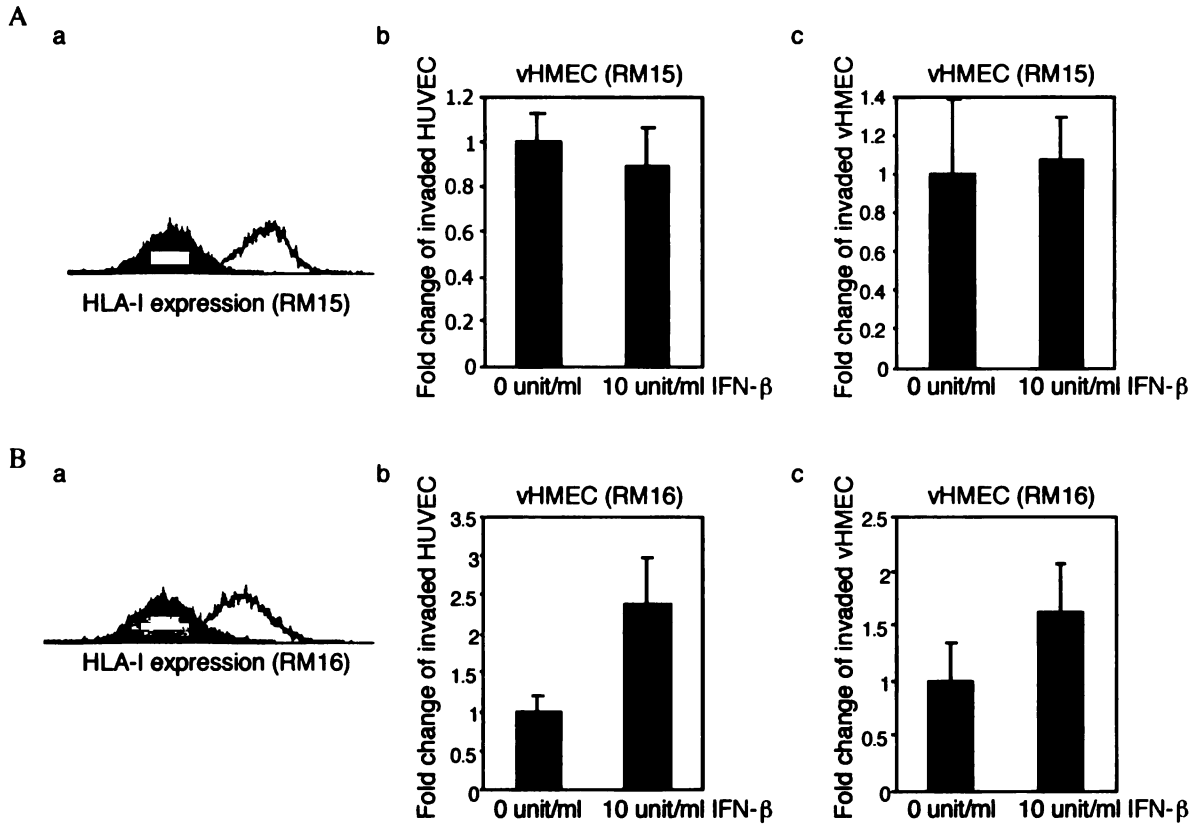


Non-specific IgG-FITC staining

DX17-FITC staining

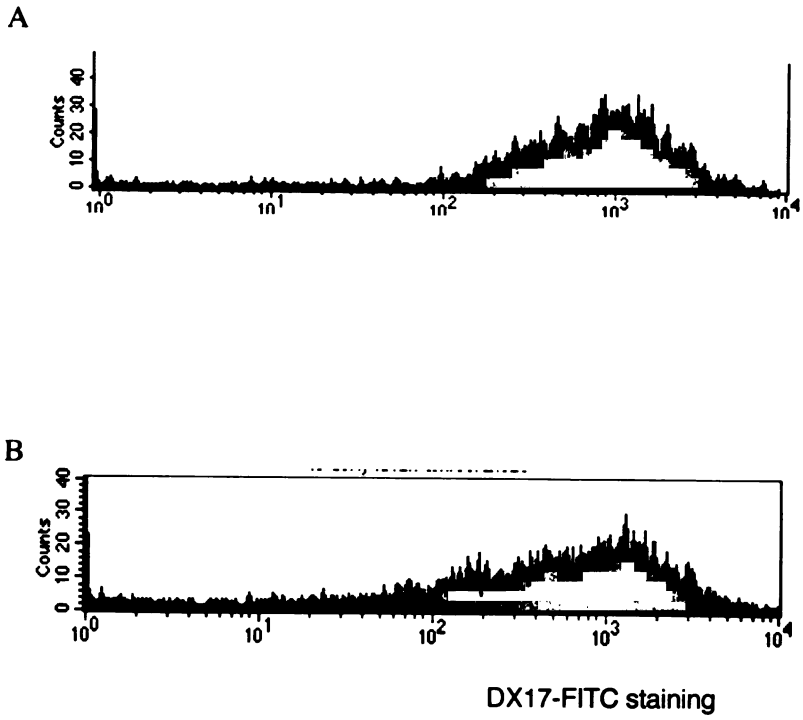
Supplementary Figure 2-S1. Interferon β (IFN-β) induces HLA-I expression. vHMECs were stained with either non-specific IgG-FITC or DX17- FITC (gift from Dr. Lewis Lanier at UCSF) for 30 minutes on ice, washed, and then subjected to flow analysis. A, non-specific IgG-FITC staining. B, DX17-FITC staining for cell surface protein HLA-I. HLA-I expression level in vHMEC after the addition of IFN-β at 0 (dark blue), 5 (green), 10 (pink), 50 (light blue), and 100 (orange) unit/ml for indicated times. Top, middle, and bottom panels represent 2, 4, and 6 days after IFN-β addition respectively.

Supplementary Figure 2-S 2



Supplementary Figure 2-S2. Activation of interferon response does not reduce HUVEC and vHMEC invasion. a, vHMEC from RM15 (A) and RM16 (B) that were exposed to IFN-β at 10 units/ml (green) for 36 hours up-regulated HLA-I expression compared to control cells (0 unit/ml IFN-β, blue). b, Fold changes in HUVEC invasion phenotype in the presence or absence of 10 units/ml IFN-β. c, Fold changes in vHMEC invasion phenotype in the presence or absence of 10 units/ml IFN-β. b and c, experiments were done as described in the experimental procedures except 10 units/ml IFN-β was added 24 hour prior to the 12-hour assay. Fold changes were determined when compared to control cells with 0 unit/ml IFN-β, which was set to be one.

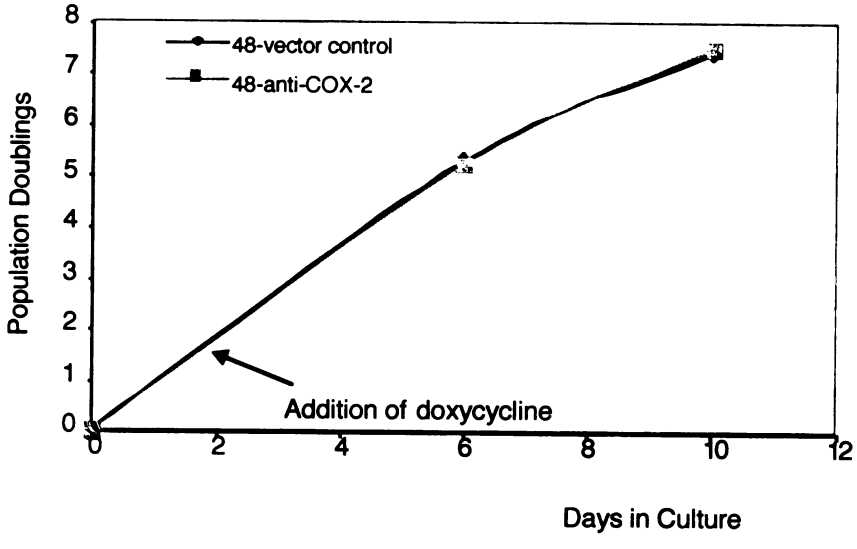
Supplementary Figure 2-S 3



Supplementary Figure 2-S3. HLA-I expression levels are similar in cells containing either a COX-2 anti-sense or a vector control construct. A, HLA-I expression level in vector control cells. B, HLA-I expression level in cells expressing COX-2 anti-sense.

Supplementary Figure 2-S 4

A



Supplementary Figure 2-S4. Growth curves are similar for vHMEC with or without COX-2 anti-sense expression. The expression of COX-2 anti-sense RNA was induced upon doxycycline (2 μg/ml) addition and the proliferation kinetics were measured for 10 days.

Chapter 3

Conditionally Replicative Adenovirus, Onyx411, Preferentially Eliminates Premalignant Variant Human Mammary Epithelial Cells

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Abstract

Breast tissues from healthy women contain a subpopulation of variant human mammary epithelial cells (vHMEC). Unlike the majority of human mammary epithelial cells (HMEC) isolated from the same tissue explants, vHMEC exhibit *p16^{INK4a}* promoter hypermethylation and extended proliferation, and acquire genomic abnormalities and COX-2 mediated premalignant phenotypes. Moreover, vHMEC-like cells exhibiting both *p16^{INK4a}* promoter hypermethylation and intense COX-2 expression exist *in vivo* in histologically normal human mammary epithelium and are candidates as precursors to breast cancer. In this study, we find that vHMEC or HMEC with down-regulated p16^{INK4a} expression exhibit higher E2F activity than HMEC. This relatively high E2F activity supports the preferential replication of conditionally replicative adenovirus (Onyx 411) and permits selective lyses of these cells by Onyx 411. These data coupled with the premalignant nature of vHMEC suggest that using conditionally replicative adenovirus such as Onyx 411may be an effective method for preventing or delaying breast cancer development.

Introduction

Breast tissues from healthy women contain a subpopulation of variant human mammary epithelial cells (vHMEC) (Brenner et al., 1998; Crawford et al., 2004; Holst et al., 2003; Romanov et al., 2001). In culture, vHMEC (post M0, post-selection HMEC, or post-senesce HMEC) exhibit different characteristics from the majority of human mammary epithelial cells (HMEC or pre-selection HMEC) isolated from the same reduction mammoplasty tissues of disease free women. Unlike HMEC, vHMEC exhibit extended proliferation (30-50 population doublings) beyond the time when HMEC enter the irreversible p16-mediated proliferation arrest, before reaching a second population growth plateau, termed agonescence (Brenner et al., 1998; Romanov et al., 2001; Tlsty et al., 2001). Nearly 100% of vHMEC approaching agonescence experience telomeric dysfunction and acquire chromosomal abnormalities including aneuploidy, polyploidy, telomeric associations, and various other classes of structural abnormalities similar to those seen in the premalignant and malignant lesions of the breast (Romanov et al., 2001; Tlsty et al., 2001).

In addition to extended proliferation and genetic alterations, vHMECs also possess epigenetic alterations (DNA hypermethylations) at the *p16^{INK4a}* locus that silence p16^{INK4a} expression (Foster et al., 1998; Huschtscha et al., 1998). p16^{INK4a} is an important cell cycle regulator and a tumor suppressor. Down-regulation of p16^{INK4a} due to either genetic (mutations and deletions) or epigenetic (DNA hypermethylations) alterations has been seen in many human tumors including those of the breast (Esteller et al., 2001; Merlo et al., 1995; Ruas and Peters, 1998). Conversely, ectopic expression of p16^{INK4a} in breast cancer cell lines that are null for p16^{INK4a} activity causes cell cycle arrest (Craig et

al., 1998; Foster et al., 2001). In vHMEC, the absence of $p16^{INK4a}$ expression is necessary for the extended proliferation that eventually leads to telomeric dysfunction and genomic instability. It is also necessary for the disruption of microtubule checkpoint control and the uncoupling of centrosome duplication cycle from DNA replication cycle that produces abnormal numbers of centrosomes per cell (Holst et al., 2004; McDermott et al., 2004; Zhang et al., 2004).

As vHMEC proliferate in culture, they up-regulate cyclo-oxygenase 2 (COX-2). In human breast cancers, over-expression of COX-2 is frequently observed (Half et al., 2002; Soslow et al., 2000; Wulfing et al., 2003) and correlates with poor prognosis (Denkert et al., 2004; Ristimaki et al., 2002; Tan et al., 2004). In murine mammary gland, over-expression of COX-2 sufficiently leads to mammary gland carcinogenesis (Liu et al., 2001), whereas inhibition of COX-2 activity reduces mammary tumor frequency in carcinogen treated animals (Abou-Issa et al., 2001; Harris et al., 2000; Kubatka et al., 2003). In vHMEC *in vitro*, over-expression of COX-2 protein specifically contributes to the expression of premalignant phenotypes including increased prostaglandin synthesis, angiogenic potential, invasive ability, and decreased apoptosis (Crawford et al., 2004).

Importantly, cells with both hypermethylated $p16^{INK4a}$ promoter sequences and intense COX-2 expression exist not only *in vitro*, but also *in vivo* in foci of histologically normal human mammary tissues from a fraction of women not yet exhibiting any indications to breast cancer (Crawford et al., 2004; Holst et al., 2003). This observation coupled with the premalignant nature of vHMEC suggests the hypothesis that vHMEC

may be *bona fide* precursors to breast cancer and eradication of vHMEC *in vivo* may prevent the occurrence of premalignant or ultimately malignant lesions of the breast.

Using conditionally replicative adenoviruses is a relatively new approach in treating neoplastic lesions (Alemany et al., 2000; Curiel, 2000; Curiel and Rancourt, 1997; Kirn et al., 2001). Theoretically, this therapy takes advantage of complementing or permissive factors specifically in tumor cells for viral replication. Hence, the therapeutic effects resulting from host cell lyses instigated by the viral replication are largely limited to the tumor cells. Furthermore, newly produced viruses in complementing or permissive host cells are competent for next round of infection and thus the therapeutic effects are amplified. Thus far, various modified adenoviruses have been generated that target cells with specific molecular alterations (Alemany et al., 2000; Cuevas et al., 2003; Davydova et al., 2004; Johnson et al., 2002; Wirth et al., 2003). In this study, we explored the possibility of utilizing conditionally replicative adenovirus, Onyx 411, for selectively lysing premalignant vHMEC.

Unlike the wild-type adenovirus, the E1a gene in Onyx 411 contains a deletion at the CR2 domain, which mediates RB binding and inactivation (Dyson et al., 1992; Fattaey et al., 1993; Johnson et al., 2002). Consequently, the E1a-ΔCR2 protein can no longer circumvent RB's inhibition on the cell cycle. Furthermore, two essential early viral genes, E1 and E4, in Onyx 411 were engineered to be under the control of exogenous human E2F1 promoters (Johnson et al., 2002). Therefore, the expressions of E1 and E4, both of which are necessary for viral replication and propagation, depend on host cell E2F activity. Hence, the lytic effects of Onyx 411 resulting from viral

replication and propagation will be largely limited to cells with high E2F activity, a consequence of a deregulated RB-E2F pathway.

Here, we present evidence indicating that vHMEC exhibit higher E2F activity when compared to HMEC, consistent with the absence or presence of $p16^{INK4a}$ expression that modulates RB-E2F pathway. Furthermore, this difference in E2F activity permits preferential replication of engineered adenovirus, Onyx 411, in vHMEC compared to HMEC and allows selective lyses of vHMEC by Onyx 411. Down-regulation of p16 expression in HMEC sufficiently leads to increased expression of the E2F target gene cyclin A and increased replication of Onyx 411 suggesting the significance of $p16^{INK4a}$ in regulating E2F activity. Our data demonstrate that engineered conditionally replicative adenovirus such as Onyx 411 may be an effective therapeutic agent for preventing or delaying breast cancer development by selectively lysing premalignant variant human mammary epithelial cells.

Results

HMEC and vHMEC Differ in $p16^{INK4a}$ Expression and E2F Activity

One characteristic that distinguishes vHMEC from HMEC is the absence of $p16^{INK4a}$ expression due to its promoter hypermethylation (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998; Romanov et al., 2001). $p16^{INK4a}$ is an important member of the cell cycle inhibitors. Its cell cycle inhibitory activity stems from its ability in modulating RB pathway by inhibiting cyclin D/cdk4/6. Cyclin D/cdk4/6 phosphorylates and thus negatively regulates RB, which inhibits E2F activity (Sherr and McCormick, 2002; Sherr and Roberts, 1995; Sherr and Roberts, 1999). Consistently, we found that vHMEC with no $p16^{INK4a}$ expression exhibited higher levels of cyclin A, one of the down-stream indicators of cellular E2F activity, than HMEC with $p16^{INK4a}$ expression (Figure 3-1A). To test whether vHMEC with no $p16^{INK4a}$ expression exhibit increased E2F activity when compared to HMEC with $p16^{INK4a}$ expression, we carried out microarray analysis focusing on E2F target genes in HMEC vs. in vHMEC since the levels of mRNA transcripts from E2F target genes are indicative of E2F activity. mRNAs from both HMEC and early or mid passage vHMEC (RM15, 21, and 48) were utilized for the microarray analysis. Both $p16^{INK4a}$ and COX-2 were included in the array as controls. As expected, microarray analysis indicated that vHMEC display lower $p16^{INK4a}$, but higher COX-2 mRNA than HMEC (data not shown). Furthermore, we found that numerous E2F target genes, including but not limited to cyclin E, follistatin, Ki-76, and ribonucleotide reductase, were up in either early or mid passage vHMEC when compared to HMEC (Figure 3-1B and C). This result indicated that vHMEC exhibit higher E2F activity than HMEC (Figure 3-1A). Representative microarray results

were validated by Q-PCR (Figure 3-1D). Results from Q-PCR confirmed the results from mircroarray experiments. On average, vHMEC expressed 5.8, 3.6, 2.4, and 2.5 fold more mRNA for cyclin E, follistatin, Ki-67, and ribonucleotide reductase respectively than HMEC. Together, our results from western analysis, microarray analysis, and Q-PCR all demonstrated that vHMEC, which lack $p16^{INK4a}$ expression due to its promoter hypermethylation, exhibited higher E2F activity when compared to HMEC with $p16^{INK4a}$ expression. We postulated that this difference in E2F activity between HMEC and vHMEC would permit differential replication of conditionally replicative adenovirus, onyx 411, that targets cells with deregulated high E2F activity.

Both HMEC and vHMEC Can be Infected by Engineered Adenoviruses

Adenovirus infects cells via receptor-mediated endocytosis (Meier and Greber, 2004; Wickham et al., 1993). The receptor for adenovirus is CAR (coxsackie and adenovirus receptor, a tight junction membrane protein) (Bergelson et al., 1997; Cohen et al., 2001). Human mammary epithelial cells in matrigel express CAR (McDermott and TDT unpublished data). To test whether HMEC and/or vHMEC can be infected by adenovirus, we infected both HMEC and vHMEC (RM21, 9, and 15) (Figure 3-2A, arrow indicates the passages at which HMEC and vHMEC were infected) with a GFP targeted replication defective adenovirus at various multiplicity of infection (MOI) ranging from 0 to 60 (Figure 3-2B). The frequency of adenoviral infection in HMEC or vHMEC was determined by the percentage of HMEC or vHMEC expressing GFP from the adenoviral vector. We found that both HMEC and vHMEC from multiple individuals could be infected by adenovirus. Furthermore, with increasing MOI, there were

increased number of HMEC and vHMEC expressing GFP (Figure 3-2B). We also observed that the infection rate varied among individuals (RM21, 9, and 15) and between HMEC and vHMEC (RM21) (Figure 3-2B).

Only Onyx 411, Among Various Engineered Adenoviruses Tested, Replicate Preferentially in vHMEC

Since both HMEC and vHMEC could be infected by adenovirus, we next subjected them to various modified adenoviruses including wild type (dl309), replication defective (dl312), and Onyx 015, 150, and 411 (Figure 3-3A). dl309 does contain deletions in the E3B region. However, it behaves as wild type adenovirus. dl312 harbors deletions in the essential viral gene, E1a, as well as in E3 and thus is a replication incompetent variant of adenovirus (Johnson et al., 2002). Onyx 150 and 411 are modified variants of the E1A-ΔCR2 mutant adenovirus, *dl922/947*, with the replacement of E1a promoter (Onyx 150) or both E1a and E4 promoters (Onyx 411) with the human E2F1 promoter (Johnson et al., 2002). Theoretically, both viruses target cells with deregulated E2F activity with more stringent dependence of Onyx 411 on cellular E2F activity than that of Onyx 150. A bar graph depicting the construct of Onyx 411 is shown in Figure 3-3B. Onyx 015 displays deletions in the *E1B55k* gene, which among other functions antagonizes p53 (Bischoff et al., 1996; McCormick, 2003). We postulated that Onyx 411 or possibly Onyx 150, both of which target cells with deregulated E2F activity, but not Onyx 015, might preferentially replicate in and thus selectively lyse vHMEC. To test this hypothesis, we exposed HMEC and vHMEC from RM15 and RM21 to dl309, Onyx 015, 150, 411, and dl312 at MOI of 5. The ability of adenovirus to lyse the host cells correlates with viral replication, which can be measured by the levels of both early

(E1a) and late (hexon, penton, and fiber) viral protein expression. We found that wild type virus (dl309) robustly replicated in both HMEC and vHMEC (Figure 3-3B and C, lanes 1 and 6, and lanes 11 and 17) whereas replication defective virus (dl312) did not (Figure 3-3B and C, lanes 5 and 10, and lanes 15 and 20). Similar results for both positive (wild type, dl309) and negative control virus (replication defective, dl312) were found in subsequent experiments (see below). Furthermore, we found that Onyx 411 preferentially replicated in vHMEC (RM15 and 21) as judged by strong early and late viral protein expressions at various time points tested (Figure 3-3C and D, lane 2 vs. lane 7, lane 12 vs. lane 17). Interestingly, Onyx 150, which also targets cells with deregulated E2F activity, replicate equally in both HMEC and vHMEC (RM15 and 21) (Figure 3-3C and D, lane 4 vs. lane 9, lane 14 vs. lane 18). Furthermore, the replication of Onyx 150 in HMEC/vHMEC was less robust than that of Onyx 411 (Figure 3-3C and D, lane 2 vs. lane 4, lane 7 vs. lane 9, lane 12 vs. lane 14, and lane 17 vs. lane 19). These results indicated that additional E2F regulation of E4 expression in Onyx 411 is required for the preferential replication of Onyx 411 in vHMEC. Onyx 015, which doesn't depend on cellular E2F activity for the expressions of its essential viral proteins, preferentially replicates in HMEC (RM15 and 21) (Figure 3-3C and D, lane 3 vs. lane 8, lane 13 vs. 18). Therefore, Onyx 411 was the only engineered adenovirus tested that preferentially replicated in vHMEC. Moreover, the HMEC populations used for the experiments shown in Figure 3-3C and D were mixed with some emerging vHMEC. Further, confluency was reached for vHMEC at later time point. These two circumstances would tend to reduce the selective effect, however, the replication preference of Onyx 411 in vHMEC remained.

Onyx 411 Preferentially Propagates in and thus Selectively Lyse vHMEC

We next focused on testing the selective killing effect of Onyx 411 in HMEC vs. vHMEC (RM 9 and 21) at various MOI of 5, 25, and 50. Similar results were obtained using HMEC/vHMEC from different individuals and at different MOI (Figure 3-4A, C, and data not shown). In all cases, we found that both early and late viral proteins from Onyx 411 expressed earlier and stronger in vHMEC than in HMEC at various time points tested (Figure 3-4A, lane 2 vs. lan5, lane 8 vs. lane 11, and lane 14 vs. lane 17; Figure 3-4C, lane 2 vs. lane 5, lane 10 vs. lane 11, lane 14 vs. 17, and lane 22 vs. lane 23; and data not shown). The levels of viral protein expression are indicative of Onyx 411's ability to replicate. Therefore, our data indicated that Onyx 411 replicated more efficiently in vHMEC vs. in HMEC at various MOI tested. Additional results from immunocytochemistry experiments supported this conclusion (Figure 3-4B). While robust expressions of late viral proteins were observed in both HMEC and vHMEC infected with wild type virus (dI309), regardless of the $p16^{INK4a}$ expression (Figure 3-4B, top row), robust expressions of both early and late viral proteins were largely limited to Onyx 411 infected vHMEC with negligible $p16^{INK4a}$ expression (Figure 3-4B, middle and lower right panels). Conversely, very limited early and late viral proteins were expressed in Onyx 411 infected HMEC with high levels of $p16^{INK4a}$ expression (Figure 3-4B, middle and lower left panels). Unlike the epithelial cells, negligible expression of early or late viral proteins was observed in isogenic human mammary fibroblasts (HMF, RM21) exposed to wild type, Onyx 411, or replication defective viruses. This data indicated that

minimal infection occurred in HMF since negligible viral proteins from wild type adenovirus were expressed. Hence, cell lysis effects of Onyx 411 were cell type and context dependent.

In addition to viral protein expression, we also determined host cell-killing effects of various adenoviruses by measuring viral titer in the media after the host lyses (Figure 3-5A, B, and data not shown). We found that viral titers were consistently higher in media from vHMEC infected with Onyx 411 than in media from HMEC infected with Onyx 411 at all time points tested (Figure 3-5A and B). This data indicate that Onyx 411 not only replicates more efficiently in vHMEC but also lyses vHMEC more efficiently than HMEC at a low MOI of 5. Wild-type virus, on the other hand, robustly and indiscriminatively lysed both HMEC and vHMEC as judged by high levels of viral protein expression and high viral titers detected in media from both HMEC and vHMEC (Figure 3-4A, C, and Figure 3-5B). Moreover, at an early time point of 24 hours, cytopathic effects (cells appear rounded up and flattened) were already apparent in both HMEC and vHMEC infected by wild-type adenovirus and in vHMEC infected by Onyx 411 (Figure 3-5C, left top and middle panel and data not shown), whereas minimal morphology changes occurred in cells infected by replication defective adenovirus or in HMEC infected by Onyx 411 (Figure 3-5C, left middle and bottom panels and data not shown). At the 48-hour time point, however, cytopathic effects in vHMEC infected with Onyx 411 were apparent whereas negligible cytopathic effects were observed in HMEC infected with Onyx 411 (Figure 3-5C, right middle panel and data not shown). Taken together, our data demonstrated that Onyx 411 preferentially replicated in and thus

selectively lysed vHMEC at various MOI tested despite the fact that HMEC (RM 21) might be more ready infected by Onyx 411 than vHMEC (RM 21) (Figure 3-2B).

Down Regulation of $p16^{INK4a}$ in HMEC Confers Increased Propagation of Onyx 411 in HMEC.

One major characteristic that distinguishes vHMEC from HMEC is the absence of $p16^{INK4a}$ expression in vHMEC due to its promoter hypermethylation (Foster et al., 1998; Huschtscha et al., 1998). This absence of $p16^{INK4a}$ expression is necessary for the emergence of vHMEC from HMEC population at their proliferative arrest and for vHMEC extended proliferation (Zhang et al., 2004). Furthermore, microarray and Q-PCR experiments examining E2F target gene expression indicated that vHMEC exhibited higher E2F activity than HMEC. Since $p16^{INK4a}$ is an important modulator of RB pathway, which regulates E2F activity, we reasoned that the lower E2F activity in HMEC was due to the presence of $p16^{INK4a}$ expression. To test this hypothesis, we down regulated $p16^{INK4a}$ through RNA interference using shRNA p16-1 (Narita et al., 2003; Paddison and Hannon, 2002). We found that the expression of small hairpin RNA for $p16^{INK4a}$ in HMEC (RM21 and 9) was effective in down regulating $p16^{INK4a}$ expression (Figure 3-6A and B). Both immunocytochemistry and western analysis demonstrated a substantial reduction in $p16^{INK4a}$ expression in cells that expressed shRNA p16-1, but not in cells that expressed the control vector. This decrease in $p16^{INK4a}$ expression in HMEC expressing shRNA p16-1 was accompanied by increased Cyclin A expression, indicating that down-regulation of $p16^{INK4a}$ expression increased E2F activity. We next subjected HMEC containing either control vector or shRNA p16-1 vector to adenoviral infection

with Onyx 411. Burst analysis demonstrated higher viral titer in media from HMEC with shRNA p16-1 than from HMEC with control vector at both 36 and 48-hour time points (Figure 3-6C). Similarly, western analysis indicated that both early and late viral proteins were expressed more robustly and earlier in HMEC expressing shRNA p16-1 than in HMEC expressing control vectors (Figure 3-6D, lane 2 vs. lan4, lane 6 vs. lane 7, and lane 9 vs. lane 11, and Figure 3-6E, lane 2 vs. lane 5, lane 8 vs. lane 11, and lane 14 vs. lane 17). We reasoned that efficient propagation of Onyx 411 in HMEC with shRNA p1-6 was not likely due to the interferon response mediated by small hairpin RNA (27 nt) since adenovirus itself represses the interferon response. Furthermore, no increase in EIF-2α phosphorylation, an indicator of the interferon response, was observed in cells infected with either control vector or vector containing small hairpin RNA (data not shown). Hence, our data indicated that just as in vHMEC with negligible p16 expression, Onyx 411 preferentially replicates in and thus selectively kill HMEC expressing small hairpin RNA for $p16^{INK4a}$. This result suggested that the level of $p16^{INK4a}$ expression in HMEC vs. vHMEC dictated the efficiency of Onyx 411 replication.

Material and Methods

Cell and tissue culture

Isolation and culturing of HMEC/vHMEC and HMF in modified MCDB 170 (MEGM, BioWhittaker, Walkersville, MD) and RPMI (Mediatech Inc, Herndon, VA) have been described previously (Hammond et al., 1984; http://www.lbl.gov/~mrgs/). HMEC/vHMEC studied in this work were derived from the reduction mammoplasty specimens of three disease free individuals, RM9, 15, and 21, in our laboratory. HMEC/vHMEC (48) were gifts from the Stampfer laboratory. HMF 21 was cultured from filtrates of reduction mammoplasty (RM21) and maintained in RPMI/10% FCS. Population doublings (PD) were calculated using the equation, PD = log (*A*/*B*)/log2, where *A* is the number of cells harvested and *B* is the number of cells initially plated.

Total RNA isolation, microarray experiment, and Q-PCR

Total RNA for HMEC and vHMEC from three different healthy donors (RM15, 21, and 48) were isolated using Qiagene kit and the corresponding cDNA were generated using invitrogene cDNA kit. mRNAs were harvested at PD 3, 9, and 30 for HMEC, early, and mid vHMEC from RM15, at PD 8.4, 27.6, and 40.3 for HMEC, early, and mid vHMEC from RM48, and at PD 0.6, 10, and 19 for HMEC, early, and mid vHMEC from RM21.

For microarray experiments, double stranded cDNA was synthesized from total RNA, purified, and used as templates to produce biotin-labeled cRNA via *in vitro* transcription. The quality/quantity of RNA/DNA for purified biotin-labeled cRNA was determined on an Agilent Bioanalyser 2100. A detailed protocol is available online at:

http://www.gladstone.ucsf.edu/gladstone/files/genomicscore/GCore_Protocol.pdf. cRNA was hybridized to Affymetrix HG U133A gene-expression chips at the University of California, San Francisco, J. Gladstone Genomics core. Data were analyzed using Affymetrix MAS 5.1 software and normalized using Robust Multichip Analysis (RMA) algorithms.

Q-PCR experiments were performed on an ABI Prism 7700 sequence detection system (ABI, Foster City, CA). DNase treated total RNAs were reverse transcripted into cDNA using random hexamer primers according to the manufacturer's instruction (TaqMan Reverse Transcription reagents, ABI). 50 ng cDNA was used for each gene analysis and experiments were done in triplicate via real time PCR using the TaqMan master mix (ABI). The levels of ribosomal 18S (PDAR 18S, ABI) and GUS (β-glucuronidase) transcripts were used for normalization. All assays were optimized to exhibit > 90% efficiency according to standard procedures using TaqMan master mixture (ABI). The measurement of each gene transcript was also tested on a serial dilution to confirm > 90% efficiency of the reaction.

Adenovirus infection, flow cytometry, and burst analysis

HMEC and vHMEC in 6 well plates (about 60-70% confluent) were exposed to adenoviruses at various MOI for either 4 hours or overnight in 1ml of MEGM growth media. Infection was stopped by washing cells with PBS, followed by replacement with MEGM growth media. Cells infected with GFP targeted replication defective adenovirus were harvested 24 hours after the initial infection via trypsinization and percent cells express GFP were determined by flow cytometry analysis using cells with no adenoviral

infection as negative control. Samples for burst assay were harvested by scraping cells in their 1 ml growth media at various time points after adenoviral infection. Amounts of adenoviruses in the media were quantified on 293/E4/pIX cells using an Elisa assay as previously described (Johnson et al., 2002).

Western analysis and immunocytochemistry

For detecting expressions of early (E1a) and late (hexon, penton, and fiber) viral proteins, 10 μg of protein lysate (in 2% SDS, 150nM NaCI with complete protease inhibitors) were used for the western analysis. E1a was detected using M73 monoclonal antibody from Dr. E. Harlow's laboratory (Massachusetts General Hospital Cancer Center) at a 1:3000 dilution. Late viral capsid proteins (hexon, penton, and fiber) were detected using rabbit polyclonal antibody for Ad5 (Access BioMedical Diagnostic Research Laboratories, Inc) at a 1:10,000 dilution. For detecting p16 and Cyclin A, 20 μg of protein lysate were used for the western analysis. p16 and Cyclin A were detected using mouse monoclonal antibodies (AB-4, 16P04 and Ab-6, CYA06) from NeoMarkers (Fremont, CA) at 1:500 and 1:100 dilutions respectively. For p16, E1a, and Ad5 immunocytochemistry, HMEC/vHMEC cells were fixed in 4% paraformaldehyde at RT for 30min and stored at 4 °C. Monoclonal p16 antibody (IgG1, Ab-1, DCS-50.1/A7, NeoMarkers), monoclonal E1a antibody (M73, 3mg/ml), and polyclonal Ad5 antibody (Access BioMedical Diagnostic Research Laboratories, Inc) were used at 1:200, 1:100, 1:300 dilutions respectively to detect p16, E1a, and late viral proteins expression.

Expression of small hairpin RNA for p16

The retroviral construct carrying a p16-specific short hairpin RNA (shRNA p16-1) under the control of U6 promoter and its control retroviral vector pMSCV were gifts from Drs. Greg Hannon and Scott Lowe at Cold Spring Harbor. The shRNA 16-1 consists inverted repeats of 27 bp corresponding to nt 381-407 of the human $p16^{INK4a}$ (CDKN2A) cDNA and an 8nt spacer separating the repeats (Narita et al., 2003; Paddison and Hannon, 2002). Both shRNA 16-1 and the vector control were transfected into (using Qiagen Effectene reagent) and packaged in phoenix-A cells grown in DMEM/10% FCS. Polybrene at a final concentration of 2 μg/ml was added to the phoenix-A cells 48 hours after the initial transfection and the medium containing retrovirus was filtered and mixed with MEGM at 1:1 ration. The mixture was added to HMEC/vHMEC and cells were kept in virus containing media for 4 to 6 hours. Two consecutive infections were carried out for each experiment. Selection for HMEC/vHMEC containing the retroviral vectors was achieved via addition of 2 μg/ml puromycin onto growth medium 72 hours after the initial infection.

Discussion

The Absence of p16 Expression is Necessary for the Presentation of Premalignant Phenotypes in vHMEC

Breast tissues isolated from reduction mammoplasties of healthy women with no indication or predisposition to breast cancers contain variant subpopulation of human mammary epithelial cells. Unlike the majority HMEC from the same tissue explants, variant HMEC do not arrest at the permanent proliferative arrest, rather, they continue to proliferate in culture (Romanov et al., 2001; Yaswen and Stampfer, 2002). Furthermore, as vHMEC continue to proliferate in culture, they experience telomeric dysfunction and acquire genomic and centrosomal abnormalities (McDermott et al., 2004; Romanov et al., 2001) as well as COX-2 mediated premalignant phenotypes including increased PGE_2 synthesis, angiogenic potential, invasive ability, and decreased apoptosis (Crawford et al., 2004). Each of these phenotypes seen in vHMEC is either direct or indirect consequence of the absence of $p16^{INK4a}$ expression.

$p16^{INK4a}$ in an important member of cell cycle inhibitors. It negatively regulates cell cycle via inhibiting cyclin D/cdk4/6. Cyclin D/cdk4/6 phosphorylates and thus inactivates RB, which inhibits E2F activity (Sherr and Roberts, 1995; Sherr and Roberts, 1999). Loss of the *$p16^{INK4a}$* locus (*CDKN2a*) has been frequently detected in many human cancers (Kamb et al., 1994a; Nobori et al., 1994). Individuals with germline point mutation in $p16^{INK4a}$ are highly susceptible to familial melanoma (Gruis et al., 1995; Hussussian et al., 1994; Kamb et al., 1994b). Specifically in mammary gland, loss of $p16^{INK4a}$ is detected in about 30% of breast cancers (Sherr and McCormick, 2002) and in 40-60% of breast cancer cell lines (Kamb et al., 1994a). Somatic inactivation of $p16^{INK4a}$

can be the result of both genetic (point mutations and deletions) and epigenetic (promoter DNA hypermethylations) alterations (Esteller et al., 2001; Merlo et al., 1995; Ruas and Peters, 1998). In addition to the absence of $p16^{INK4a}$ expression in tumor cells, aberrant cytoplasmic expression of $p16^{INK4a}$ is also seen in 9% of invasive (n= 368) and 4% of intraductal carcinomas (n=52) (Emig et al., 1998; Geradts and Wilson, 1996). Conversely, reintroducing $p16^{INK4a}$ into breast cancer cell lines that are null for p16 activity causes cell cycle arrest (Craig et al., 1998; Foster et al., 2001). The importance of $p16^{INK4a}$ in human cancers was further strengthened by the finding that alterations in Cdk4 (either a point mutation that disrupts the binding of $p16^{INK4a}$ and thus render it $p16^{INK4a}$ resistant or gene amplification) and Cdk6 (over-expression) are also detected in human cancers (Tang et al., 1999; Wolfel et al., 1995; Zuo et al., 1996). Taken together, these data clearly point to a role of $p16^{INK4a}$ in suppressing human tumor development.

In vHMEC, the absence of $p16^{INK4a}$ is both necessary and sufficient for their continued proliferation beyond the time when HMEC are in proliferative arrest (Zhang et al., 2004). Consequent to extended proliferation, vHMEC gradually experience telomeric dysfunction, acquire genomic abnormalities, and undergo sustained subsequent induction of COX-2 (Crawford et al., 2004; Romanov et al., 2001). The absence of $p16^{INK4a}$ expression in vHMEC is also responsible for the accumulation of abnormal numbers of centrosomes (McDermott et al., 2004) and for defects in cell cycle checkpoint control in response to disrupted microtubules (Holst et al., 2004). Therefore, this initial defect in cell cycle check point control at least in part due to epigenetic modulations of $p16^{INK4a}$ promoter may play a deterministic role for the expression of vHMEC's premalignant

phenotypes and is necessary for the evolution of vHMEC into premalignant, may be even malignant state.

p16 Modulates E2F Activity in HMEC/vHMEC

p16 inhibits cyclin D/cdk4/6 that inhibits RB, a negative regulator of E2F activity (Sherr and Roberts, 1999). Unlike HMEC, vHMEC do not express p16 due to its promoter hypermethylation (Foster et al., 1998; Huschtscha et al., 1998). Hence, we hypothesized that vHMEC might exhibit higher E2F activity than HMEC expressing $p16^{INK4a}$. Indeed, we found that E2F activity inversely correlates with $p16^{INK4a}$ expression in HMEC/vHMEC. In HMEC expressing $p16^{INK4a}$, the mRNAs or protein levels of E2F target genes, which are indicative of E2F activity, were consistently lower than those in vHMEC with no $p16^{INK4a}$ expression as measured by western analysis, microarray analysis, and Q-PCR. Furthermore, the expression of $p16^{INK4a}$ in HMEC plays a direct role in modulating E2F activity since down-regulation of $p16^{INK4a}$ expression in HMEC via shRNA was sufficient to increase the expression of E2F target gene. Taken together, our data indicate that $p16^{INK4a}$ dictates E2F activity in HMEC and that the difference in E2F activities between HMEC and vHMEC was likely due to the presence or absence of $p16^{INK4a}$ expression.

Cells Comparable to vHMEC Exist *in vivo*

The two molecular markers that are specific for vHMEC in culture are $p16^{INK4a}$ promoter hypermethylation and COX-2 over-expression (Crawford et al., 2004; Romanov et al., 2001). We reasoned that should vHMEC exist *in vivo*, we would be able to identify cells that exhibit both markers in breast tissues from normal individuals with

no indication or predisposition to breast cancers. Indeed, we have previously identified an *in vivo* population of mammary epithelial cells in histologically normal breast tissue that simultaneously displays $p16^{INK4a}$ promoter hypermethylation and intense COX-2 staining (Crawford et al., 2004; Holst et al., 2003). The co-localization of intense COX-2 staining and $p16^{INK4a}$ promoter hypermethylation within the same mammary epithelium indicates that cells with vHMEC characteristics exist *in vivo*. These data coupled with the premalignant nature of vHMEC *in vitro* suggests that this *in vivo* population of mammary epithelial cells has the potential for extended proliferation permitted by the absence of $p16^{INK4a}$ expression and to express premalignant phenotypes mediated by COX-2. Therefore, they may be relevant to human disease and may be *bona fide* precursors for breast cancer.

Consistent with our precursor hypothesis, we found that the majority (~85%) of premalignant breast lesions, ductal carcinoma in situ (DCIS), over-expresses COX-2 (Shim et al., 2003). Intriguingly, regardless of the COX-2 status in the DCIS lesions, nearly all of the adjacent normal-appearing mammary epithelial cells surrounding the lesions over-express COX-2 (Shim et al., 2003). These data suggest that normal-appearing epithelial cells surrounding DCIS may have already engaged in the disease process and may provide field effects for the emergence of premalignant lesions. Furthermore, epidemiological studies indicate that long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin, which inhibit COX-2, reduces breast cancer risk (Harris et al., 2003). Taken together, these data suggest that variant human mammary epithelial cells with premalignant phenotypes exist *in vivo* prior to the presentation of premalignant or malignant lesions and that selective elimination of this *in*

vivo population may be of critical importance in preventing or delaying breast cancer development.

Employing Engineered Adenovirus to Selectively Eliminate vHMEC

Given that vHMEC exhibit extended proliferation and premalignant phenotypes similar to those seen in malignant cells and given that vHMEC-like cells exist *in vivo*, it is important to selectively eliminate vHMEC and prevent them from further progression. In this report, we demonstrated that engineered adenovirus, Onyx 411, was effective in preferential elimination of vHMEC.

Adenoviruses have the ability to infect, propagate, and lyse host cells. The newly engineered conditionally replicative adenoviruses also exhibit required specificity for targeting cells with specific cellular alterations (Alemany et al., 2000). In general, two different approaches have been applied to modify adenovirus to achieve cancer cell specificity (Alemany et al., 2000; Barnes et al., 2002). One way is to employ tumor specific promoters to drive the expression of essential viral genes (Brunori et al., 2001; Cuevas et al., 2003; Davydova et al., 2004; Doronin et al., 2001; Fuerer and Iggo, 2002; Hallenbeck et al., 1999; Kurihara et al., 2000; Wirth et al., 2003). Another way is to take advantage of defects in cell cycle regulatory functions seen in cancer cells. Onyx 411 and dl922-947 (E1a-ΔCR2) are examples in this category (Fueyo et al., 2000; Heise et al., 2000; Johnson et al., 2002). Both of these adenoviruses contain deletions in E1a-ΔCR2, which no longer binds to and inhibit RB. Hence, the replications of these two adenoviruses are largely limited to cells with existing RB pathway defects.

We tested the ability of Onyx 411, 150, and 015 to selectively eliminate vHMEC in this study. We found that Onyx 411, which targets cells with deregulated E2F activity, was the only engineered adenovirus that preferentially targeted vHMEC over

HMEC. This finding is consistent with the observation that vHMEC exhibit high E2F activity and no $p16^{INK4a}$ expression. It is important to note that Onyx 411 was able to replicate in HMEC with low E2F activity as demonstrated by viral protein expressions at later time points and by low viral titer detected in the media at later time points. These data indicate that the low E2F activity in HMEC was able to permit Onyx 411 replication. However, when compared to vHMEC, replication of Onyx 411 in HMEC was much slower and less robust. This differential replication of Onyx 411 in HMEC vs. vHMEC allows preferential elimination of vHMEC by the adenovirus. Hence, Onyx 411 is capable of selectively eliminating vHMEC and preventing vHMEC from further progression. Furthermore, the replication of Onyx 411 was largely limited to the epithelial cells, not the isogenic fibroblasts. Since breast cancers are largely derived from cancerous mammary epithelial cells, Onyx 411 may cause very limited effects on adjacent fibroblasts.

In summary, out data indicate that, in addition to extended proliferation and premalignant phenotypes, vHMEC also differ from HMEC in E2F activity. This differential in E2F activity permits preferential replication of Onyx 411 in vHMEC, which leads to selective lysis of vHMEC. Should vHMEC prove relevant to human disease, our data implicate that employing engineered adenoviruses such as Onyx 411 or its future derivatives is a reasonable approach to target mammary epithelial cells with premalignant phenotypes for breast cancer prevention rather than intervention.

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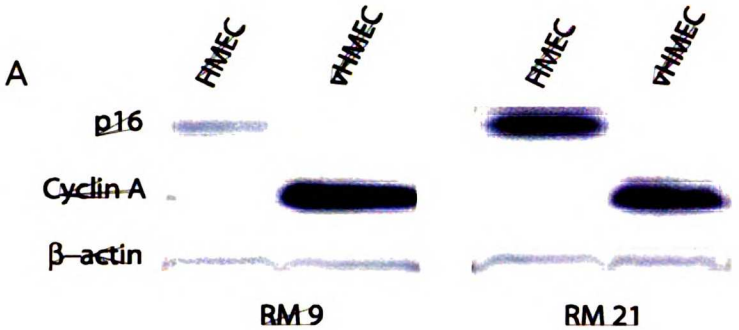
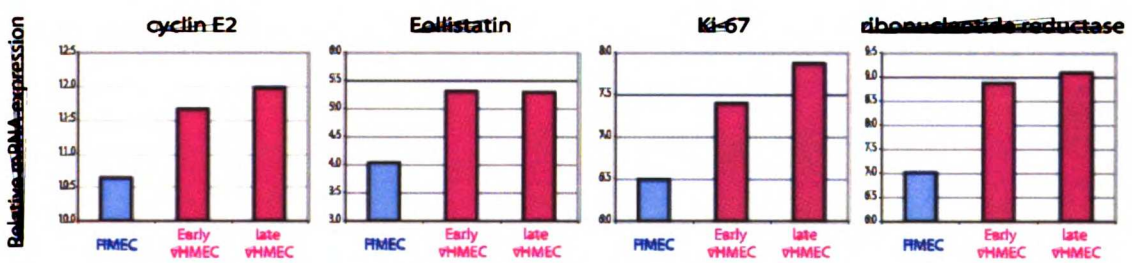
Figure 3 - 1



B

C



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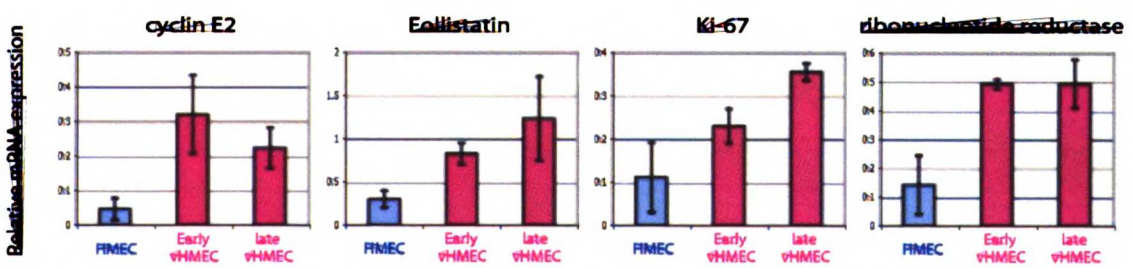


Figure 3-1. vHMEC without $p16^{INK4a}$ expression exhibit higher E2F activity than HMEC expressing $p16^{INK4a}$. A, Western analysis for p16 and cyclin A expression in HMEC and vHMEC from RM 9 and 21 (PD 3 and 14, and PD 0.5 and 14 respectively). Levels of β-actin were used as loading control. B, Clustering of E2F target gene expressions in HMEC, early, and mid vHMEC. mRNA from HMEC, early and late vHMEC of three different individuals (RM15, 21, and 48) were applied to the microarray experiments that focused on E2F target genes. C, Bar graphs indicating relative mRNA levels (obtained from microarray experiment) for cyclin E, follistatin, Ki-67, and ribonucleotide reductase in HMEC, early and mid vHMEC. D, Q-PCR results of relative mRNA levels for cyclin E, follistatin, Ki-67, and ribonucleotide reductase in HMEC, early and mid vHMEC. Results shown here are average mRNA levels in HMEC, early and mid vHMEC from three different individuals (RM15, 21, and 48). Y error bar represents S.E.M.

Figure 3 - 2

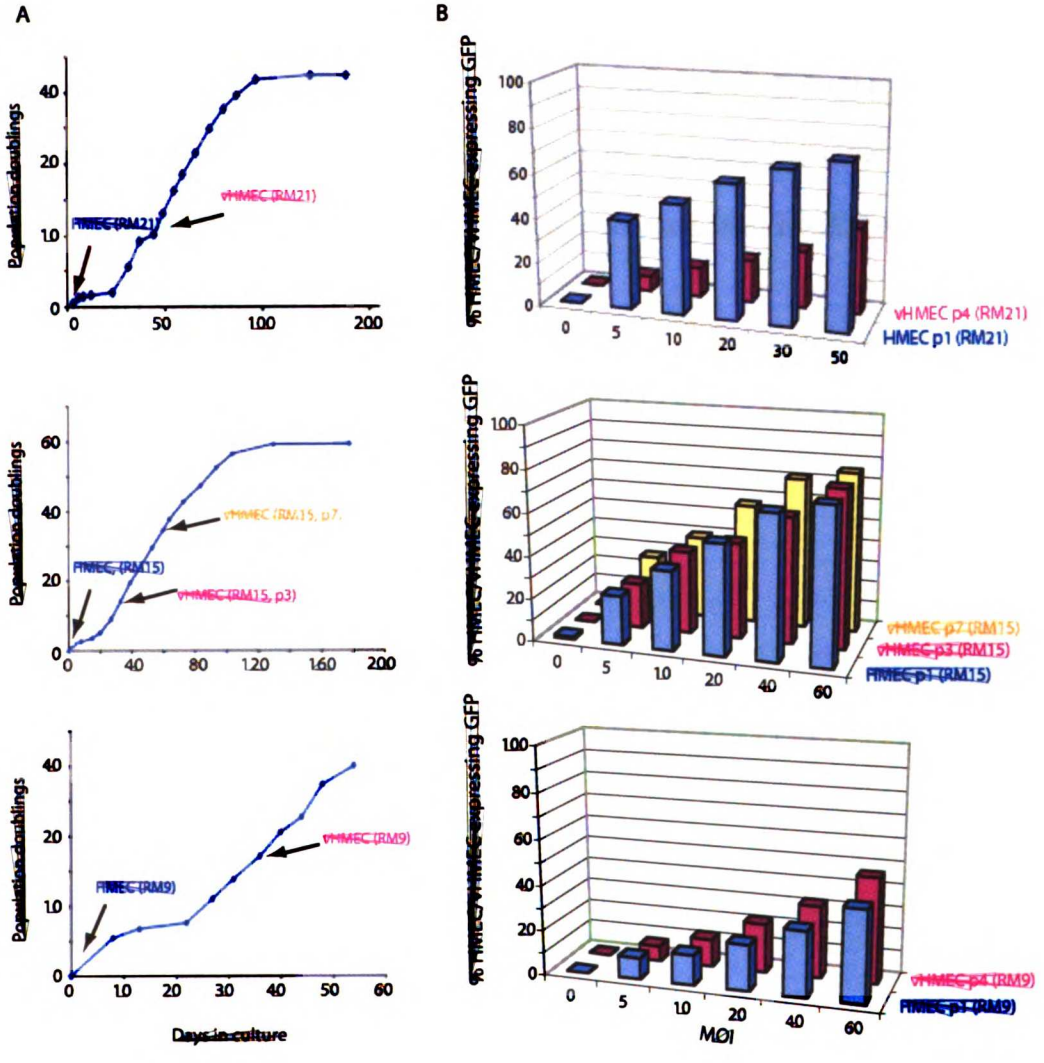

A
B
Population doublings
Days in culture
vHMEC (RM21)
HMEC (RM21)
vHMEC (RM15, p7)
HMEC (RM15)
vHMEC (RM15, p3)
vHMEC (RM9)
HMEC (RM9)
% HMEC/vHMEC expressing GFP
vHMEC p4 (RM21)
HMEC p1 (RM21)
vHMEC p7 (RM15)
vHMEC p3 (RM15)
HMEC p1 (RM15)
vHMEC p4 (RM9)
HMEC p1 (RM9)
MOI

Figure 3-2. Adenovirus infects both HMEC and vHMEC. A, Growth curves for HMEC/vHMEC (RM 9, 15, and 21). Arrows indicate the passages when adenoviral infection occurred for this experiment. B, Adenovirus infects all HMEC and vHMEC tested. Cells were exposed to GFP-tagged replication defective adenovirus for 4 hours. Percent cells expressing GFP from viral vector 24 hours after the initial exposure indicates percent cells infected with adenovirus. MOI indicates multiplicity of infection. Variable infection rates were observed for HMEC and vHMEC from different individuals. At a low MOI of 5, about 40% HMEC and 7% vHMEC from individual RM21, 10% HMEC and 7% vHMEC from individual RM9, and 20% HMEC or vHMEC from individual RM15 expressed GFP.

Figure 3 - 3

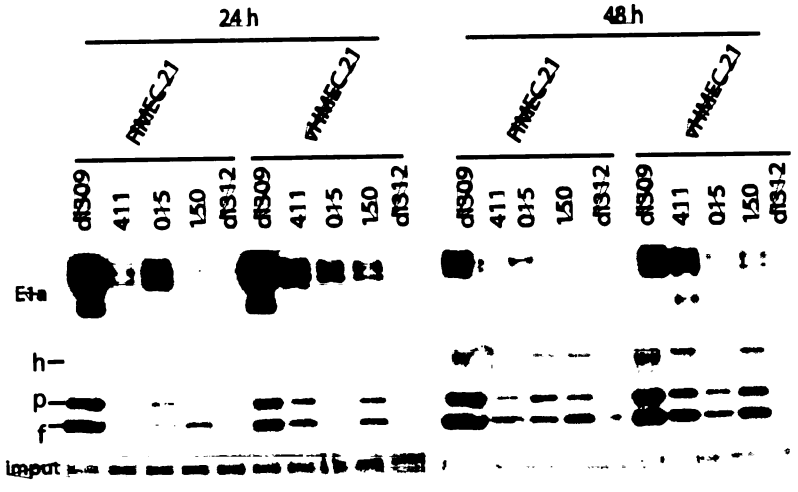
A

Ad5 virus	E1a promoter	E1a	E1b-55k	E3b region	E·
dl309			Δ		
dl312	Δ	Δ	Δ		
Onyx 015			Δ	Δ	
Onyx 150	E2F-1	ΔCR2	Δ		
Onyx 411	E2F-1	ΔCR2	Δ	E2F-1	

B

Onyx 411 Construct

C



D

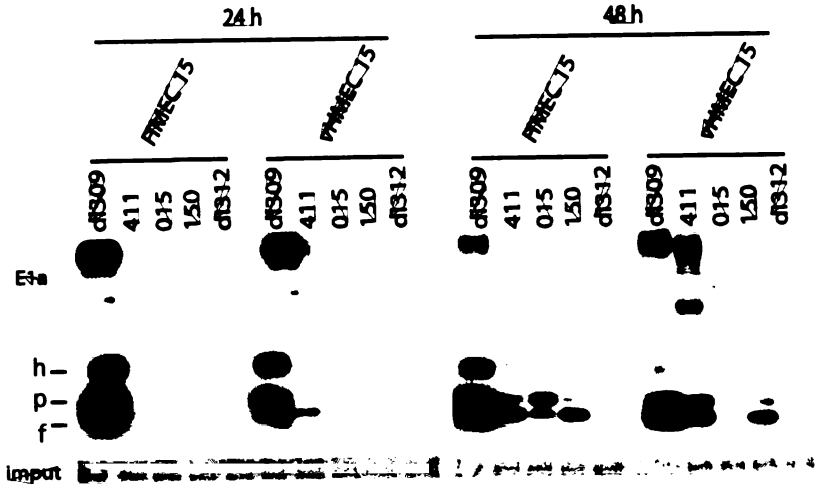


Figure 3-3. Onyx 411, among all adenoviruses tested, replicates more efficiently in vHMEC than in HMEC. A, Table of all adenoviruses examined in this study. All viruses are derived from an Ad5 background. Regions of mutations specific to each virus are indicated on the top of the table. Δ represents deletions. Small deletion in E1a CR2 domain (from nucleotides 922 to 947) is depicted as E1a-ΔCR2. B, A bar graph depicts the construct of Onyx 411. C, Expressions of early (E1a) and late (hexon (h), penton (p), and fiber (f)) viral proteins in HMEC or vHMEC (RM21, PD 1 and 11 respectively) infected with adenoviruses at MOI of 5. Time points of 24 and 48 hours were included in the western analysis. Coomassie staining of total proteins were used as loading control. D, Expressions of early and late viral proteins in HMEC and vHMEC (RM15, PD 2 and 13 respectively) infected with adenoviruses at MOI of 5. Time points of 24 and 48 hours were included in the western analysis. Coomassie staining of total proteins were used as loading control.

Figure 3 - 4

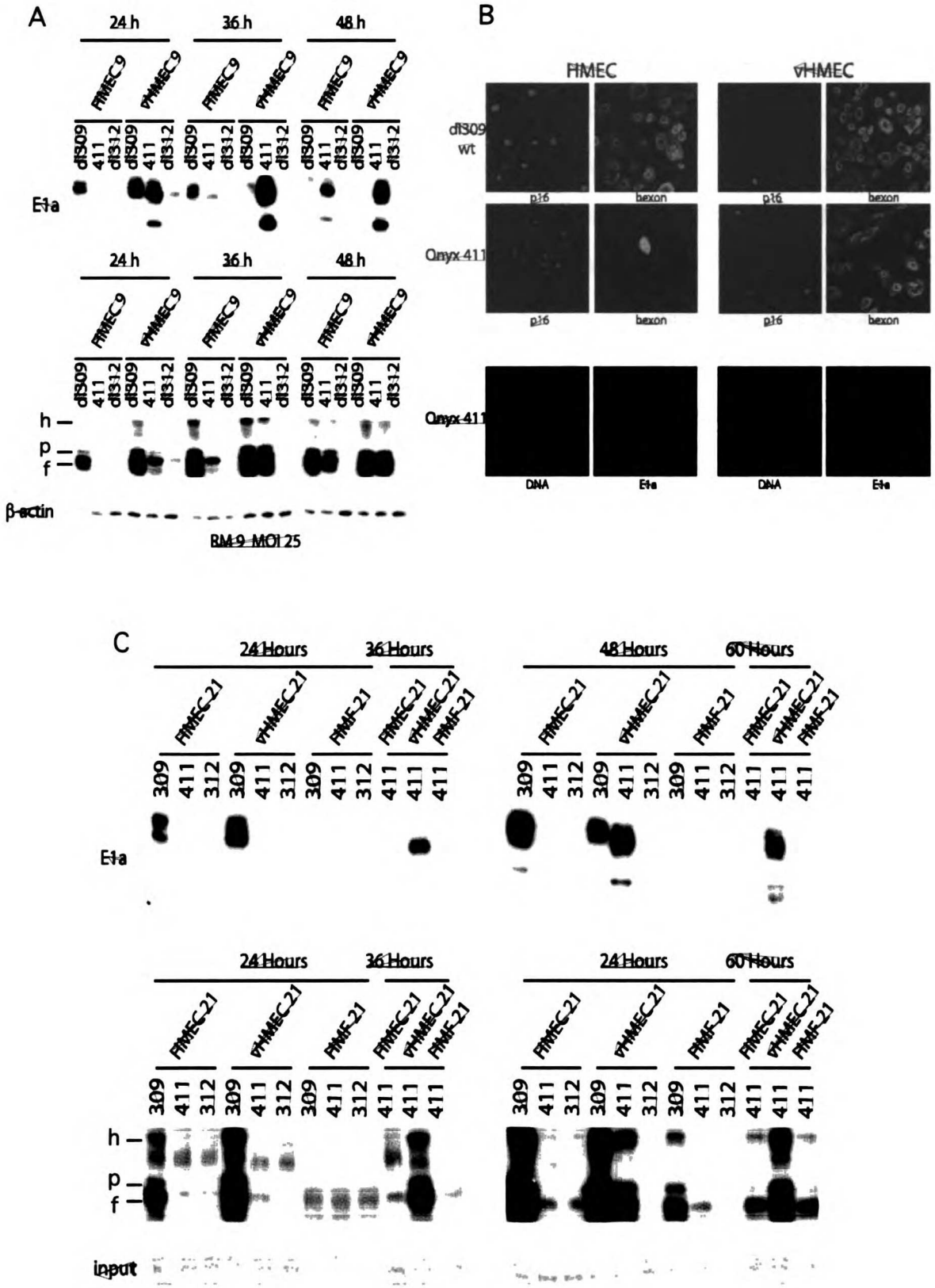
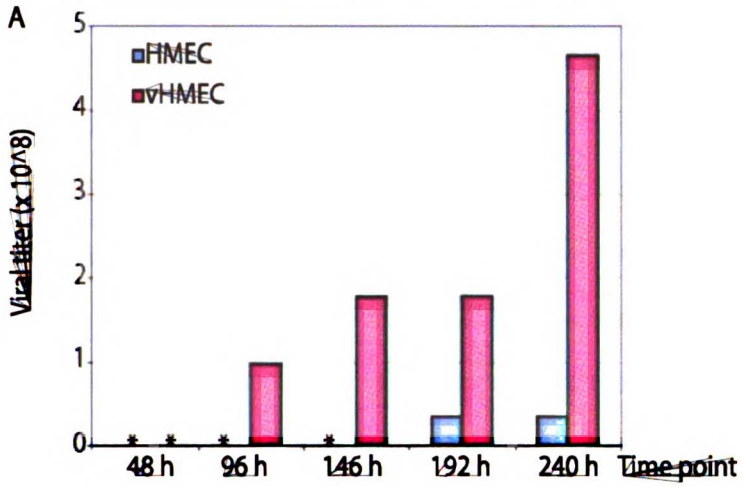

A
24 h
36 h
48 h
FHMEC-9
vHMEC-9
dl309
411
dl312
E1a
h
p
f
β-actin
RM 9 MOI 25
B
FHMEC
vHMEC
dl309
wt
Onyx-411
p16
bexon
DNA
E1a
C
24 Hours
36 Hours
48 Hours
60 Hours
FHMEC-21
vHMEC-21
FHMF-21
309
411
312
E1a
h
p
f
input

Figure 3-4. Onyx 411 preferentially replicates in vHMEC. A, Expressions of early and late viral proteins in HMEC and vHMEC (RM9, PD 3 and 14 respectively) infected with wild type, Onyx 411, and control viruses at MOI of 25. Time points of 24, 36, and 48 hours were included in the analysis. β-actin levels were used as loading control. B, Immunocytochemistry for the expressions of p16 and viral proteins in HMEC and vHMEC (RM9, PD 3 and 14 respectively) infected with adenoviruses at MOI of 25. C, Expressions of early and late viral proteins in HMEC, vHMEC, and HMF (RM21, PD 0.5, 14, and 3 respectively) infected with wild type, Onyx 411, and control viruses at MOI of 5. Time points of 24, 36, 48, and 60 hours were included in the western analysis. Similar results were obtained when HMEC and vHMEC (RM21, PD 0.6 and 19.6 respectively) were infected with adenoviruses at MOI of 50 (data not shown).

Figure 3 - 5

A



B

Time point	Viral Titer					
	wt virus		Onyx 411		defective virus	
	FHMEC	vHMEC	FHMEC	vHMEC	FHMEC	vHMEC
48 hours	45.2	1.33	*	*	*	*
96 hours	43.8	81.6	*	0.97	*	*
146 hours	63.8	48.4	*	1.77	0.43	*
192 hours	43.6	50.7	0.34	1.78	*	*
240 hours	1.49	1.29	0.34	4.65	*	*

C

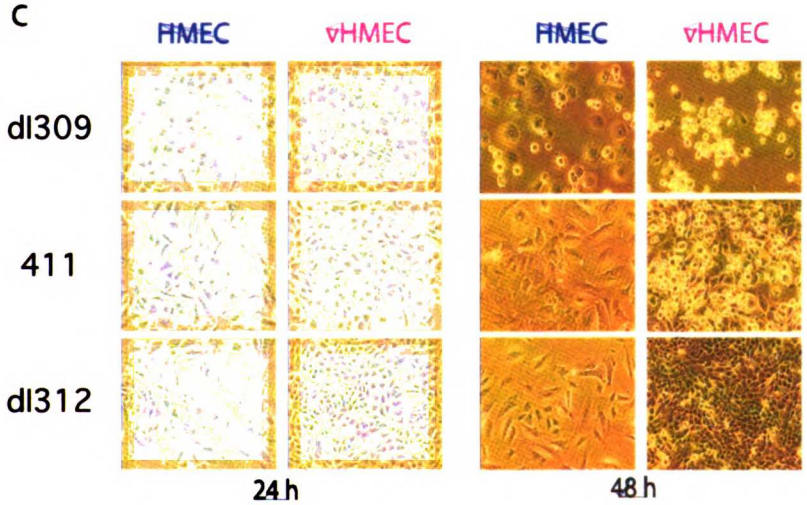


Figure 3-5. Onyx 411 preferentially lyses vHMEC. A, Burst analysis for viral load released in the media after host cell lysis. HMEC and vHMEC (RM21, PD 3.4 and 8.1 respectively) were infected with Onyx 411 at MOI of 5. * indicates viral titers were below the level of detection. B, Viral titers measured in media from HMEC and vHMEC (RM21, PD 3.4 and 8.1 respectively) infected with wild type, Onyx 411, and replication defective viruses at MOI of 5. Similar results were obtained in experiments using HMEC (PD 2.6) and vHMEC (PD 34.6) from RM15 (data not shown). * indicates viral titers were below the level of detection. C, Cytopathic effects of adenoviruses in HMEC vs. vHMEC (RM9). HMEC (PD 3) and vHMEC (PD 14) were infected with adenoviruses at MOI of 5. At 24-hour time point, cytopathic effects were seen in cells infected with wild-type virus and in vHMEC infected with Onyx 411. These cells appeared rounded-up and flattened. At 48-hour time point, lytic effects were observed in wild-type virus infected cells and in vHMEC infected with Onyx 411. Similar cytopathic effects were observed in experiments using cells from different RM (RM15, and 21) or with different MOI (MOI of 25 or 50) (data not shown).

Figure 3 - 6

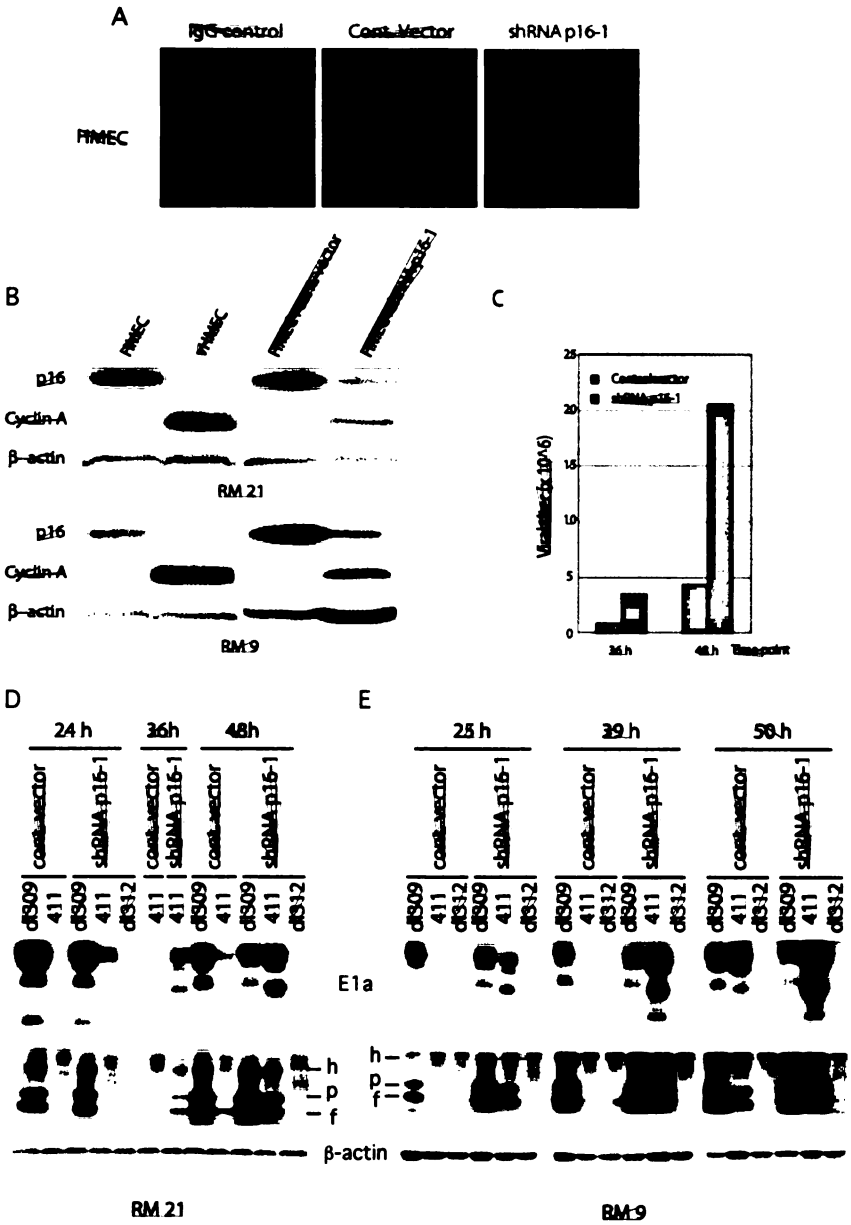

A
IgG-control
Cont. Vector
shRNA p16-1
HMEC
B
HMEC
HMEC
p16
Cyclin-A
β-actin
RM 21
p16
Cyclin-A
β-actin
RM 9
C
Cont.vector
shRNA p16-1
36 h
48 h
D
24 h
36h
48h
cont.vector
shRNA p16-1
dl309
411
dl312
E1a
h
p
f
β-actin
RM 21
E
25 h
39 h
50 h
cont.vector
shRNA p16-1
dl309
411
dl312
h
p
f
β-actin
RM 9

Figure 3-6. Down-regulation of p16 expression in HMEC confers efficient replication of Onyx 411. A, Immunocytochemistry results for p16 expression in HMEC containing either control vector or shRNA p16-1. Control experiment was done using non-specific IgG1 as primary antibody. B, Western analysis for p16 and cyclin A expression in HMEC, vHMEC, and HMEC containing either control vector or shRNA p16-1. Expression of small hairpin RNA for p16 in HMEC (RM9 and 21) sufficiently down-regulated p16 protein expression. C, Burst analysis for viral titers produced in Onyx 411 infected HMEC containing either control vector or shRNA expressing vector. Timer points of 36 and 48 hours were included in the analysis. D and E, western analysis for early and late viral protein expressions in HMEC (RM9 and 21) containing either control vector or vector expressing shRNA for p16.

Chapter 4

Conclusions

Yongping G. Crawford

Abstract

The following conclusions can be drawn from this dissertation study. First, increasing numbers of vHMEC over-express COX-2 as they propagate *in vitro* and approach agonescence. Second, this over-expression of COX-2 proteins specifically contributes to the premalignant phenotypes seen in vHMEC including increased PGE_2 synthesis, angiogenic potential, invasive ability, and decreased apoptosis. Third, DNA damage is sufficient to induce COX-2 over-expression in vHMEC. Fourth, vHMEC like cells exhibiting both $p16^{INK4a}$ promoter hypermethylation and intense COX-2 expression exist *in vivo* in histologically normal human mammary tissues. Fifth, premalignant vHMEC can be selectively eliminated upon exposure to either COX-2 specific inhibitors or engineered adenovirus that targets cells with deregulated E2F activity. In the current chapter, I will elaborate on these points as well as findings by other members in our laboratory and speculate on the implications of these findings.

Discussion and Conclusions

Studies employing human mammary epithelial cells isolated from healthy women with no indication or predisposition to breast cancer have provided valuable insights into the early steps of breast carcinogenesis (Crawford et al., 2004; Holst et al., 2003; Romanov et al., 2001; Tlsty et al., 2001; Yaswen and Stampfer, 2001; Yaswen and Stampfer, 2002). Human mammary epithelial cells isolated from normal individuals contain two distinct populations with regard to the presence or absence of an epigenetic modulation at the $p16^{INK4}$ locus (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998). The variant population (vHMEC) does not express p16, an important cell cycle inhibitor and tumor suppressor, due to its promoter hypermethylation (Foster et al., 1998; Huschtscha et al., 1998; Romanov et al., 2001; Tlsty et al., 2001). As a result, vHMEC are able to proliferate beyond a p16-mediated growth arrest that the majority HMEC experience in culture. As vHMEC continue to proliferate, they begin to experience telomeric dysfunction and acquire substantial amounts of genomic abnormalities similar to those seen in early steps of breast cancer (Romanov et al., 2001; Tlsty et al., 2001). Hence, we hypothesize that vHMEC may represent a precursor population that is primed for breast carcinogenesis.

If vHMEC are precursors for breast cancers, they will satisfy the following descriptions. First, unlike normal human mammary epithelial cells, vHMEC exhibit premalignant phenotypes. Second, vHMEC must exist not only *in vitro* but also *in vivo* in breast tissues either containing or devoid of cancer lesions. Third, vHMEC must behave differently from normal mammary epithelial cells in response to certain oncogenic insults

both *in vitro* and *in vivo*. In other words, vHMEC should be more susceptible to oncogenic transformation than normal human mammary epithelial cells.

Studies from Romanov *et al.* have indicated that vHMEC exhibit premalignant phenotypes including extended proliferation and genomic instability (Romanov et al., 2001; Tlsty et al., 2001). Moreover, studies from McDermott *et al.* demonstrated that vHMEC not only exhibit genomic abnormalities but also centrosomal abnormalities including structure abnormalities and abnormal numbers of centrosomes per cell (McDermott et al., 2004). Both genomic and centrosomal abnormalities are phenotypes frequently seen in malignant cells and are thought to be driving forces for malignant transformation. Not so surprisingly, Dr. Holst, Zhang, and McDermott in our laboratory find that all of the above malignant-like phenotypes seen in vHMEC are linked to epigenetic modulations at the promoter region of an important tumor suppressor, p16 (Holst et al., 2004; McDermott et al., 2004; Zhang et al., 2004).

The significance of p16 in suppressing breast carcinogenesis originates from the following observations. First, the absence of p16 expression either due to genetic (deletion or point mutations) or epigenetic (hypermethylations) alterations has been found in 30% of breast cancers (Sherr and McCormick, 2002) and 40-60% of breast cancer cell lines (Kamb et al., 1994). Second, aberrant cytoplasmic expression of p16 is seen in additional 9% of invasive (n= 368) and 4% of intraductal carcinomas (n=52) (Emig et al., 1998; Geradts and Wilson, 1996). Third, alterations in cdk4 (either point mutations that disrupt the binding of $p16^{INK4a}$ and render it $p16^{INK4a}$ resistant or gene amplification) and cdk6 (over-expression) are also detected in human cancers (Tang et al., 1999; Wolfel et

al., 1995; Zuo et al., 1996). Fourth, in culture, reintroducing $p16^{INK4a}$ into breast cancer cell lines causes a dose-dependent cell cycle arrest (Craig et al., 1998; Foster et al., 2001).

In *in vitro* studies using human mammary epithelial cells isolated from normal individuals devoid of any breast lesions, Dr. Holst and Zhang find that the loss of p16 expression resulting from its promoter hypermethylation is both necessary and sufficient for vHMEC's extended proliferation (Zhang et al., 2004). In addition to the extended proliferation that eventually leads to telomeric dysfunction and genomic instability (Romanov et al., 2001), vHMEC also exhibit defects in the cell cycle checkpoint control in response to microtubule disruptions and in the maintenance, but not the initiation, of DNA damage-induced checkpoint response (Holst et al., 2004). Interestingly, down-regulation of p16 expression via RNA interference in HMEC recapitulates the defective microtubule response seen in vHMEC, but not the defective maintenance in DNA damage-induced checkpoint. Two conclusions can be made based on these results. First, p16 is necessary for microtubule checkpoint control, but dispensable for both the initiation and the maintenance of DNA damage-induced checkpoint control. Second, vHMEC must possess additional genetic and/or epigenetic alterations that abrogate the maintenance of DNA damage-induced checkpoint control.

In search for additional epigenetic alterations that may exist in vHMEC, Dr. Reynold and Mahvash Sigorodinia in our laboratory employed both RLGS and ROMA technologies to screen for genome wild changes in CpG methylation. They found that HOXa9 and Runx3 loci, among others, exhibit increased methylation at their promoter regions in vHMEC (personal communication). Both HOXa9 and Runx3 are transcription factors involved in development (Chen and Capecchi, 1999; Coffman, 2003). In the case

of Runx3, its tumor suppressing activity has been demonstrated in the murine model. Furthermore, loss of Runx3 expression due to either deletion or hypermethylation correlates with gastric cancers in humans (Bae and Choi, 2004; Li et al., 2002; Lund and van Lohuizen, 2002). Hypermethylation of HOXa9 has also been linked to certain tumors such as neuroblastoma (Alaminos et al., 2004) and its role in cancer development is currently under investigation by many laboratories. It is intriguing to speculate that hypermethylations at these two loci may contribute to premalignant phenotypes seen in vHMEC. The significance of HOXa9 and Runx3 promoter hypermethylations in vHMEC is under intense investigation in our laboratory. It remains interesting to test whether the loss of p16 co-operates with the absence of HOXa9 or Runx3 to dictate vHMEC phenotypes.

The exciting finding made by McDermott *et al.* in our laboratory reveals a novel function of p16 in coupling centrosome duplication with DNA synthesis in human mammary epithelial cells (McDermott et al., 2004). They find that, unlike HMEC, a substantial fraction of vHMEC is unable to prevent centrosome re-duplication when DNA synthesis is experimental stalled at the S-phase and exhibits abnormal multiple centrosomes. Furthermore, down-regulation of p16 expression in HMEC recapitulates the uncoupling phenotype seen in vHMEC and hence permits the generation of multiple centrosomes per cell in many cases, but not all. The exact mechanism through which p16 ensures accurate coupling remains to be discovered. Nevertheless, this data indicates that loss of p16 expression in HMEC is necessary, but not sufficient for centrosome re-duplication within a singe cell cycle and that the uncoupling phenotype seen in vHMEC may result from the absence of p16 expression. In the experimental situation,

centrosome re-duplication is only seen when DNA synthesis is artificially stalled upon hydroxyurea exposure. What then stalls DNA synthesis in vHMEC to allow the manifestation of multiple centrosomes per cell? The answer may lie in the observations made by Krystyna Kozakiewicz, a cytogenetist in our laboratory. She observed frequent chromosomal abnormalities including deletions, insertion, translocation, telomeric associations, and aneuploidy and polyploidy in vHMEC, but not in HMEC (Romanov et al., 2001). Perhaps, DNA damage transiently stalls DNA synthesis and creates a suitable environment for centrosome re-duplication in the absence of p16 expression.

In short, the absence of p16 expression is responsible for multiple phenotypes seen in vHMEC including extended proliferation, microtubule checkpoint defects, and the uncoupling of centrosome duplication from DNA synthesis. It is also important to note, however, that vHMEC are not simply HMEC with low levels of p16 expression. Results from Holst *et al.* indicates that vHMEC exhibit maintenance defects in DNA damage-induced checkpoint whereas HMEC with down-regulated p16 expression don't (Holst et al., 2004). Furthermore, data from Reynold *et al.* indicate that in addition to $p16^{INK4a}$ promoter hypermethylation, additional CpG methylations are seen in vHMEC that affect genes expression such as HOXa9 (Reynold et al., personal communication).

When I joined the lab, I was keenly interested in finding out what distinguished vHMEC from HMEC at the molecular level in addition to the absence of p16 expression. In collaboration with NIEHS, we employed microarray experiments to assess molecular events specifically to either vHMEC or HMEC. My interests in this experiment were as follows. First and at the least, we should be able to obtain molecular markers specific to vHMEC for their identification *in vivo*. If vHMEC are precursor cells for breast cancer,

they must exist not only *in vitro* but also *in vivo* in breast tissues either containing or devoid of cancerous lesions. Therefore, to be able to identify vHMEC *in vivo* using specific molecular markers identified from microarray experiments will be extremely helpful in supporting our hypothesis. Second, we might be able to identify key molecular events that define phenotypes of vHMEC. In other words, I wanted to investigate the molecular mechanism(s) that determines vHMEC's fate. Third, we might be able to reveal molecular target(s) for preventing vHMEC from further progression. Should vHMEC be precursors for breast cancer, selective elimination of vHMEC from HMEC population would have profound effects in breast cancer prevention.

The microarray experiments revealed a plethora of gene expression differences between HMEC and vHMEC. Among all the genes that are up regulated in vHMEC, the most prominent change is seen in the expression of cyclo-oxygenase 2 (COX-2) (Crawford et al., 2004). Additional immunocytochemistry experiments demonstrate that (COX-2) is highly up-regulated in a fraction of vHMEC, but not HMEC. This data suggest that loss of p16 expression is not sufficient to induce COX-2 expression since all vHMEC do not express p16, but only some of them over-express COX-2. We postulated that an event(s) subsequent to the absence of p16 expression might signal COX-2 up-regulation. We tested and found that DNA damage within vHMEC is sufficient in inducing COX-2 up-regulation. In addition to endogenous DNA damage signals that induce COX-2 up-regulation, exogenous factors may also influence COX-2 expression.

High level of COX-2 expression has been observed previously in many tumors including those of breast (Soslow et al., 2000). Furthermore, studies in murine models clearly ascertain its role in tumor promotion (Oshima et al., 1996). In the murine

mammary gland, over-expression of COX-2 is sufficient for mammary tumor development (Liu et al., 2001). In vHMEC *in vitro*, this up-regulation of COX-2 specifically contributes to the premalignant phenotypes seen in vHMEC including increased PGE_2 synthesis, angiogenic potential, invasive ability, and decreased apoptosis (Crawford et al., 2004). Taken together, we postulate that COX-2 up-regulation may be an early event in the malignant transformation of human mammary epithelia. Hence, understanding the molecular mechanism involving COX-2 induction will assist us to understand vHMEC evolution during the malignant transformation. To dissect the molecular mechanism involving COX-2 induction, we will need to identify the up-stream players that signal and/or mediate COX-2 expression in vHMEC and to map out the down-stream pathways of COX-2 in promoting premalignant phenotypes associated with vHMEC.

Thus far, two functionally relevant markers have been attributed to vHMEC. They are $p16^{INK4a}$ promoter hypermethylation and COX-2 over-expression. If vHMEC exist *in vivo*, it should be possible to identify epithelial cells *in vivo* that also exhibit both markers. Indeed, we found that in disease-free breast tissues from reduction mammoplasty, intense COX-2 staining co-localize with foci of $p16^{INK4a}$ promoter hypermethylation (Crawford et al., 2004; Holst et al., 2003). Conversely, mammary epithelial cells that do not exhibit $p16^{INK4a}$ promoter hypermethylation do not over-expression COX-2 (Crawford et al., 2004; Holst et al., 2003). This data suggests that cells comparable to vHMEC *in vitro* exist *in vivo*. Additional markers will certainly aid our confidence in this conclusion. Currently, our laboratory is actively testing other vHMEC specific markers identified from microarray experiments.

It is, nevertheless, valid to hypothesize that vHMEC-like cells *in vivo* may be *bona fide* precursors for breast cancer since hypermethylation at $p16^{INK4a}$ promoter may permit extended proliferation, centrosomal abnormality, and cell cycle defects, and COX-2 over-expression may contribute to the manifestation of malignant phenotypes described as the "Hallmarks of Cancer". Epidemiology studies that correlate the existence of vHMEC-like cells *in vivo* with breast cancer risk will help to address this hypothesis. However, this experiment will be very long-term and expensive. The alternative *in vitro* 3D model and xenoplants in mice may be more approachable to test this hypothesis. It remains to be tested whether vHMEC are more readily transformed than HMEC in response to certain oncogenic insults.

In a different approach, we reasoned that if vHMEC-like cells *in vivo* are premalignant, we should be able to identify them in premalignant lesions of the breast including ductal carcinoma *in situ* (DCIS). To address this question, Shim *et al.* screened 46 DCIS specimens for COX-2 over-expression (Shim et al., 2003). They found that COX-2 over-expression occurs in 85% of DCIS samples and is associated with increased nuclear grade. What's more interesting is that the adjacent normal appearing mammary epithelial cells surrounding the DCIS lesions strongly over-express COX-2. This finding suggests that the adjacent normal-appearing epithelial cells have engaged in the disease process and may provide field effects for the emergence of malignant cells. The status of COX-2 expression in early pre-malignant lesions remains to be determined. We would predict that COX-2 over-expression might also be detected in these lesions.

If additional experiments indicate that vHMEC are precursors for breast cancer, the findings in this dissertation work will have profound implications. First, COX-2

over-expression could be used as an early diagnostic marker for identifying women who have not yet manifested the full blown disease, but are at high risk for breast cancer. Second, COX-2 may also server as a therapeutic intervention target for preventing or delaying breast cancer development. Indeed, epidemiology studies have suggested that long-term usage of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, which inhibits COX-2, reduces breast cancer risk (Harris et al., 2003). Third, in addition to COX-2 inhibition, we could also exploit the difference in p16 expression and E2F activities between HMEC and vHMEC and employ engineered adenovirus to selectively eliminate vHMEC.

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