hTERT ENABLES HETEROGENEITY IN A POPULATION OF HMEC WHICH SUSTAINS ONCOGENE SENSITIVE AND RESISTANT CLONES

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Dedication

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Abstract

Mammalian cells activate multiple mechanisms of defense against uncontrolled proliferation when oncogenic mutations occur. In this way, the process of transformation relies on breaching such defenses to promote cancer. Telomere maintenance, mediated by expression of telomerase in cancer cells, is associated with evasion of proliferative senescence, a known barrier against cancer. Whether telomerase contributes to deregulate activation of other mechanisms of defense is not clear in the field. We decided to study whether expression of telomerase deregulates the activation of known mechanisms of defense by comparing primary Human Mammary Epithelial cells (HMECS) and hTERT immortalized HMECS when they express oncogenic Ras. To our surprise we found that contrary to primary cells, hTERT immortalized cells respond differently against oncogenic Ras by undergoing post-replication arrest and destructive autophagy. Although these barriers were available, the population of cells did not respond uniformly against oncogenic Ras. Instead, we observed variability in the execution of these responses. Our results suggest that the process of immortalization fosters the opportunity for evolution of the population. In these conditions many cells can harness mechanisms of defense while a minority of cells becomes resistant to them, an important characteristic for further cancer progression.

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Chapter 1

Known mechanisms of defense against cancer progression

Cancer arises by genetical and epigenetical changes affecting the genome of metazoan cells. This is an evolutionary process that that drives with time the manifestation of the neoplasm. However, despite the trillions of potential targets for developing cancer, only a few successfully develop into a tumor during the process of a lifetime. This suggests that multicellular organisms possess several mechanisms to control the manifestation of cancer[1]. Exhaustion of these checkpoints is essential for occurrence of this cancer.

Apoptosis

Apoptosis is a mechanism of cell death that is used by multicellular organisms to eliminate unwanted cells in a diversity of settings[2]. At least two programs trigger apoptosis, each regulated at different levels. The “extrinsic” cell death pathway, briefly discussed in this section, is activated by ligation of cell surface death receptors (e.g. Fas and TNF-R) with their cognate ligands. Once activated, receptors form death-inducing complexes (DISC) which relay activation of apoptosis machinery. Alternatively, the “intrinsic” pathway is the primary death program that responds to the availability of survival signals, cell stress and injury. The central target of this pathway is the integrity of the mitochondrial membranes which sequester a variety of effector molecules that promote cell death.

The integrity of the mitochondrial outer membrane is determined by the balance between pro-apoptotic and anti-apoptotic proteins[3]. Pro-apoptotic proteins Bax and Bak are in charge of disrupting the mitochondrial membrane by a process known as Mitochondrial Outer Membrane Permeabilization (MOMP). This process facilitates release of proteins

sequestered in the intermembrane space which culminates in the activation of caspases, cysteine proteases that coordinate the demolition of the cell. The process of MOMP is antagonized by anti-apoptotic BCL-2 proteins which inhibit permeabilisation functions of Bax and Bak. Both antiapoptotic and pro apoptotic proteins are positively or negatively regulated by BH3-only members (Bcl2 familiy members containing a Bcl2 homology-3 domain).

Although much is known about apoptosis regarding its role for maintaining homeostasis of tissues, apoptosis can also be activated by deregulated oncogenes. Successful cell proliferation requires two independent signals: activation of the growth machinery and suppression of the apoptotic program that is activated as a consequence of engaging cell division[4]. However, deregulated proliferation creates an intracellular imbalance between growth and survival signals, a balance carefully coordinated by extracellular factors which activate multiple signaling pathways. Therefore, when a cell senses deregulated proliferation signals it engages a suicide program as a fail-safe mechanism.

Several oncogenes have the capacity to activate apoptosis; Myc is a classic example. Myc is important for the regulation of growth and development of normal cells and its deregulated expression is very common in cancers. However Myc can induce apoptosis in certain circumstances such as serum deprivation or other types of cellular stress[5]. Although deregulated Myc causes cells to proliferate uncontrollably, it also promotes exhaustion of survival factors that are responsible for suppressing apoptosis. This suggests that cells possess mechanisms or “traps” to recognize aberrant activation of oncogenes[1, 4].

Cellular Senescence

Cellular senescence was described more than 40 decades ago when Leonard Hayflick showed for the first time that normal human cells had a limited proliferation capacity in culture[6]. The term cellular senescence was applied to this cell culture phenomenon based on the similitude that this behavior had with organismal aging, a deteriorative process that comes after development and maturation[7].

Throughout the following decades we have learned more about the causes and mechanism of cellular senescence. In fact, we are beginning to understand its physiological relevance. On the one hand, initial studies have proposed that cellular senescence is important for tumor suppression. In this way senescence has been considered beneficial for the organism by protecting it from cancer. On the other hand, recent studies suggest that cellular senescence contributes to the process of aging where tissue regeneration and repair deteriorate with age[8]. This side suggests that senescence is detrimental to the organism because it limits regeneration and repair.

What causes cellular senescence?

The initial answer came from understanding why normal mammalian cells do not proliferate indefinitely in culture. However, several recent studies have shown that many stimuli can also induce senescence[9]. In this review we will focus on two mechanisms that mammalian cells utilize for tumor suppression: telomere-dependent and telomere independent oncogene-induced senescence.

Telomere-dependent senescence

Telomeres are stretches of tandem repeats of DNA and associated proteins that cap the ends of linear chromosomes. Telomeres have two functions: protecting chromosome ends from DNA repair activities and prevent the loss of genetic information by providing

a mechanism of telomere-length maintenance [7, 10, 11]. During replication of genomic DNA, telomeres become subject to attrition due to the fact that DNA polymerase fails to replicate the terminal ends of the lagging strands, a phenomenom called “the end replication problem”[12]. **Figure 1-1**

When telomere attrition reaches a critical length their protective structure is disrupted. As a consequence, dysfunctional telomeres trigger a DNA Damage Response (DDR)[13]. DDR enables cells to sense damaged DNA, for example double strand breaks, and then halt cell-cycle progression until the damage is repaired. Many proteins participate in this process, including protein kinases (for example Ataxia Telangiectesia Mutated (ATM), adaptor proteins (for example 53BP1) and chromatin modifiers (for example γH2AX).

Recent studies demonstrate that DDR differs in telomeric and non-telomeric regions[14, 15]. On one side DNA damage at telomeres is irreparable causing persistent DDR that inactivates proliferation irreversibly. On the other hand DNA damage outside telomeric regions does not trigger senescence; instead it activates repairing machinery to terminate DDR. If the damage is reparable cells might escape proliferation arrest, but if damage is irreparable cells might proceed to death.

There is evidence, however, that oncogenes promote deregulated DNA synthesis which activates DDR in non-telomeric regions, and arrests cell proliferation irreversibly[16-18]. This phenomenon will be discussed in the next section.

Oncogene-Induced Senescence (OIS)

Activated oncogenes, mutant versions of normal genes that have the potential to transform cells in collaboration with additional mutations, can also trigger cellular senescence. While replicative senescence is activated by erosion of telomeres during cell divisions, a similar phenotype can occur in “young cells” in response to these mutant

versions of these genes. This phenomenon was observed when an oncogenic version of Ras was expressed in normal human fibroblasts[19]. Subsequently other members of the Ras signaling pathway (Raf, MEK,MOS and BRaf) as well as E2Fs proliferation inducing proteins were shown to cause senescence when overexpressed or expressed in its mutant version[7].

Seminal studies in the last six years have presented the unique relationship that exists between OIS and aberrant DDR activation. In these studies expression oncogenic Ras (in fibroblasts) promotes a burst in proliferation followed by a slowing in cell propagation and eventually cells senesce. It is at the end of this burst in proliferation where DDR activation coincides. Moreover, these studies have also shown that DDR activation has a causal role for establishing and maintaining OIS. Inactivation of genes implicated in DDR leads the cell to resume cell proliferation and predisposes the cell to transformation [16, 17]. These studies put forward the argument that DDR is recruited to suppress strong mitogenic signals and enforces senescence to inhibit cell transformation.

Although there is more to be learned about the mechanisms that lead to oncogene-induced DNA damage generation, it has been proposed that DDR activation is a result of deregulated DNA synthesis. Studies with Myc and Ras have shown that these oncogenes stimulate the firing of multiple replication forks as part of a proliferative program. These forks rapidly stall, collapse and form double strand breaks. Stalled and collapsed forks are responsible for the activation of DDR and cell-cycle checkpoints [1620].

Besides activation of DDR, it has been observed that cells that trigger OIS undergo changes in their chromatin structure which affect the expression of genes important for proliferation. Expression of oncogenic Ras in human primary fibroblasts induces chromatin changes on E2F-responsive promoters and is associated with stable

repression of E2F target genes in an Rb tumor suppressor-dependent manner[21]. Recent studies have described the players responsible for coordinating induction of heterochromatin condensation[22].

Two important tumor suppressor pathways have been implicated for the induction and maintenance of OIS: p53 and p16-pRb. The Mitogen Activated Protein Kinase (MAPK) signaling cascade appears to be the principal inducer of p16 and ARF, proteins that ultimately activate Rb and P53[23]. P53 and Rb promote senescence by controlling several effector molecules, typical examples are: p21 (a cyclin dependent kinase inhibitor) as well Promyelocytic Leukemia (PML) and other chromatin-modifying factors that produce a repressive state against aberrant mitogenic signaling. It is important to know, however, that there are both cell-type and species-specific differences in how a cell engages p53 or Rb to induce senescence [7]. Finally, it appears that senescence can be activated independently of P53 and Rb activation suggesting the possibility of an alternative senescence mechanism[24, 25].

Recent studies from Mikhail Blagosklony's lab have presented a different perspective about oncogene-induced senescence and its relation to proper coordination of cell division [26]. Demidenko et al presented the argument that senescence is a result of uncoupling of cell cycle transit and growth stimulation[27]. Simultaneous cell cycle arrest, mediated by hypermitogenic signals, and persistent growth stimulation render cells to senescence. Therefore, successful cell division requires that both cell cycle transit and cell growth work in parallel.

Insights into the molecular mechanism of this type of cell division arrest present two important molecular pathways: MAP kinase and mTOR. Mitogen-activated Protein kinase pathways stimulate cell cycle machinery by inducing Cyclins, the activators of

Cyclin Dependent Kinases (CDKs). However, activation of MAPkinase can also induce Cyclin Dependent Kinase Inhibitors (CDKIs) which inhibit the cell cycle [26]. mTOR is a metabolic sensor, activated in presence of nutrients, which coordinates cell growth. However, in the presence of cell cycle arrest, induction of mTOR promotes irreversible cell division arrest even when cell cycle transit is induced[27]. Only inhibition of mTOR and relief of cell cycle arrest can promote re-entry into cell division transit.

Physiological relevance

Much of our understanding of causes and consequences of OIS comes from cell culture studies. However, during the last six years the field of senescence has been enriched with the discovery of senescence in *in vivo* models[28]. For example, expression of endogenous oncogenic $KRas^{G12D}$ in mice has been shown to induce senescence in early stages of lung and pancreatic cancer. Also, senescence has been observed in melanocytic nevi in mice that express endogenous mutant $Braf^{V600E}$, the oncogenic version of Braf which is a downstream effector of Ras[29, 30].

Some studies using mouse models of oncogenic Ras have presented conflicting results. Studies from Tuveson's group have not found evidence of senescence in $KRas^{G12D}$ – driven lung tumors[31]. However, expression of oncogenic KRas at physiological levels has lower capacity to activate canonical effector pathways as seen *in vitro* as well prolonged latency in tumor formation[32]. This suggests that expression of oncogenic KRas at endogenous levels is not fully tumorigenic and tumor susceptibility depends on cellular context. For example, transgenic-inducible expression of $HRas^{G12V}$ in the mammary gland of mice leads to hyperproliferation when Ras is expressed at low levels while senescence is observed when the oncogene is highly expressed. The differences in levels of expression have led to the contradictory conclusion. Therefore, it is the absolute levels that are an important factor for the induction of cellular senescence.

The observations of a senescence phenotype *in vivo* have not been limited to mouse models but also have been reported in human premalignant tumors. Melanocytic nevi, a benign type of tumor associated with the presence of oncogenic $BRAF^{V600E}$ presents markers of senescence[25]. As another example, loss-of-function mutation of tumor suppressor gene NF1 underlies the familial cancer syndrome known as neurofibromatosis type 1. NF1 is a negative regulator of Ras activity and in its absence hyperactivated Ras signaling promotes formation of neoplastic lessions. Interestingly expression of senescence markers has been observed in this type of tumor [33].
These studies have helped support the hypothesis that senescence works as a tumor suppressive mechanism and is not just an *in vitro* artifact. In this way, breaching oncogene-induced senescence barrier represents an important step for transformation. As we will see soon, the activation of senescence encompasses many cellular processes which contribute to the overall irreversible cell cycle arrest.

Hallmarks of Senescence

Several markers can be used to identify senescent cells *in vitro* and *in vivo*. However no marker indentified so far is entirely specific to a senescence state. Moreover, not all senescent cells express all senescence markers. Finally, manifestation of these markers is time-dependent, requiring several days to develop. Nonetheless, senescent cells display several phenotypes, which together define the senescent state **Figure 1-2.**

Irreversible Growth Arrest

Senescent cells undergo a growth arrest which is permanent and cannot be reversed by known physiological stimuli. Lack of DNA synthesis, typically detected by 5-bromodeoxyuridine (BrdU) or by immunostaining of proteins such as Ki-67, is characteristic of this irreversible cell cycle arrest. Unfortunately, this marker does not distinguish between senescent, quiescent and post mitotic cells.

Morphological changes

Senescent cells in cell culture have an increased size (commonly named the “fried egg morphology), sometimes enlarging more than two times relative to the size of non-senescent counters[6].

Senescence-Associated β-galactosidase (SA-βgal)

SA-βgal was the first marker that is more specific for detection of senescent cells [34]. This marker is detected by histochemical staining of senescent cells. Recent studies have shown that SA-βgal derives from lysosomal β-galactosidase, reflecting an increased lysosomal biogenesis in senescent cells [35].

p16 upregulation

A specific role for p16 in OIS is suggested to be maintenance of the growth arrest through activation of Rb, crucial guardian of the Restriction Point.

Senescence Associated Heterochromatin Foci (SAHF)

Senescent cells can also be detected by cytological markers of SAHFs. SAHFs can be identified by binding of DNA dyes such as DAPI and presence of certain histone modifications which are associated with heterochromatin condensation (e.g., H3 Lys9 methylation and Heterochromatin Protein – HP1). It has been proposed that induction of SAHF, mediated by pRb tumor suppressor, regulates silencing of critical pro-proliferative genes (for example E2Fs)[21].

Senescence-Associated DNA Damage Foci (SA-DDF)

Cells that senesce by persistent DDR signaling present nuclear foci, which contain activated DDR proteins (for example: γ-H2AX and P53-binding protein 1 (53bp1) [13].

Senescence-Associated Secretory Phenotype (SASP)

Senescent cells with persistent DDR signaling secrete a plethora of factors that are involved in insulin-like growth factor (IGF), Transforming Growth Factor β (TGF β) signaling, Extracellular Matrix (ECM) remodeling and inflammatory cytokines (e.g.IL-6,IL-8) [36]. SASP factors have been shown that promote senescence in non malignant cells (in an autocrine manner), or proliferation of tumor cells (in a paracrine manner).

Role of cellular immortalization in carcinogenesis

One of the hallmarks that characterizes human cancers is the acquisition of unlimited replicative potential which contrasts with the limited life span that primary human cells possess [37]. As mentioned in earlier sections during each cycle of genomic DNA replication, telomeric DNA length is not maintained due to the phenomenon called “the end replication problem”. Exhaustion of telomeric DNA induces replicative senescence, an irreversible cell cycle arrest. In order to overcome this problem, cells possess two mechanisms to replenish telomere length: expression of telomerase enzyme and a telomerase-independent mechanism known as alternative lengthening of telomere (ALT) [38]. The latter will not be discussed in this work.

Telomerase is a reverse transcriptase that replenishes telomeric DNA located at chromosome ends. It is composed of an RNA template (TR) for synthesis of telomeric repeats, a catalytic reverse transcriptase component (TERT), and Dyskerin (DKC1). Telomerase is expressed and active in human stem/progenitor cells and germ line cells, as well as in a subset of somatic cells (e.g. activated lymphocytes) [39]. Mutations that affect the appropriate function of the telomerase holoenzyme have been found in a spectrum of human telomerase diseases, for examples of which include: dyskeratosis congenital and aplastic anemia [11]. Patients with mutations in telomerase subunits show premature telomere shortening and impaired stem cell function.

The discovery of the telomerase enzyme complemented the telomere-dependent senescence model and provided a framework to understand how cancers avoid this barrier. It was observed that human tumors and immortal cell lines had high telomerase activity and stable telomere lengths as compared to normal somatic cells[40]. However, the causal relationship between telomere length and senescence as well as the relationship between telomerase expression and cellular immortality was not yet clear. Cloning of the hTERT gene and its introduction into several human somatic cell types showed that it conferred indefinite proliferation[4142]. Therefore the relationship between reactivation of telomerase enzyme and unlimited proliferation capacity was established [40, 41, 43].

Several studies showed later, however, that expression of hTERT alone is not sufficient to promote cellular immortalization. The importance of p16/Rb and the P53/p21 pathways was identified in suppressing immortalization [42, 44-46]. P16 and p21 belong to the family of cyclin-dependent kinase inhibitors (CDKIs) that prevent inactivation of the Retinoblastoma tumor suppressor gene. Therefore, eradication of these tumor suppressive pathways and de novo expression of hTERT seems critical for promoting unlimited proliferation.

Recent studies have shown that the process of immortalization is a process that takes an extended period of time and encompasses many genomic changes besides the alterations mentioned above. The Myc gene is known for targeting hTERT promoter and consequently inducing its expression. In a seminal study, Bazarov et al showed that expression of Myc in Human Mammary Epithelial cells (HMEC) was not able to induce expression of hTERT nor rescue from telomere attrition at earlier time points [47]. However, in late passage Myc-expressing-cells, hTERT mRNA levels were upregulated and amplification of chromosome 5 regions was detected. They concluded that Myc was

not sufficient to induce hTERT expression and that additional genomic changes were needed for HMEC immortalization. They postulated that chromatin regulation is important for repressing hTERT and changes in chromatin organization (e.g. genomic instability generated by telomere attrition) provide one important step for immortalization.

There have been debates in the literature about whether telomerase is an oncogene and the process of immortalization is tumorigenic. As explained by Harley [48] telomerase does not promote deregulated growth as oncogenes or loss of tumor suppressor genes does. Evidence for this has come from studies on embryonic stem cells [49] and germline cells [40, 50], cells that express telomerase and do not show signs of deregulated growth. Also ectopic expression of hTERT on several human cells lines has not shown evidence of deregulated growth[51, 52]. However many speculations conceive the idea that telomerase expression creates an opportunity for cells to accumulate mutations that foster clones that can outcompete others and become a tumor.

Conclusions

Mammalian cells possess several barriers against tumor formation. Apotosis and senescence are the best known mechanisms of defense against cancer. However, other mechanisms of defense (e.g. tumor immunology and others not yet described) work together to keep tight control of tissue homeostasis. Therefore, cancer only manifests when these mechanisms of defense are abrogated, thereby promoting deregulated tumor growth.

Figure 1-1 The end-replication problem.

A) Synthesis of the leading strand of DNA occurs continuosly to the end of the template strand where as synthesis of the lagging strand is mediated by discontinuous Okazaki fragments initiated by labile RNA primers (black boxes). The RNA primers are degraded, gaps are filled by DNA polymerase and Okazaki fragments are ligated. B) The terminal gap of the lagging strand is no filled. These results in loss of terminal DNA sequence each time the cell replicated.

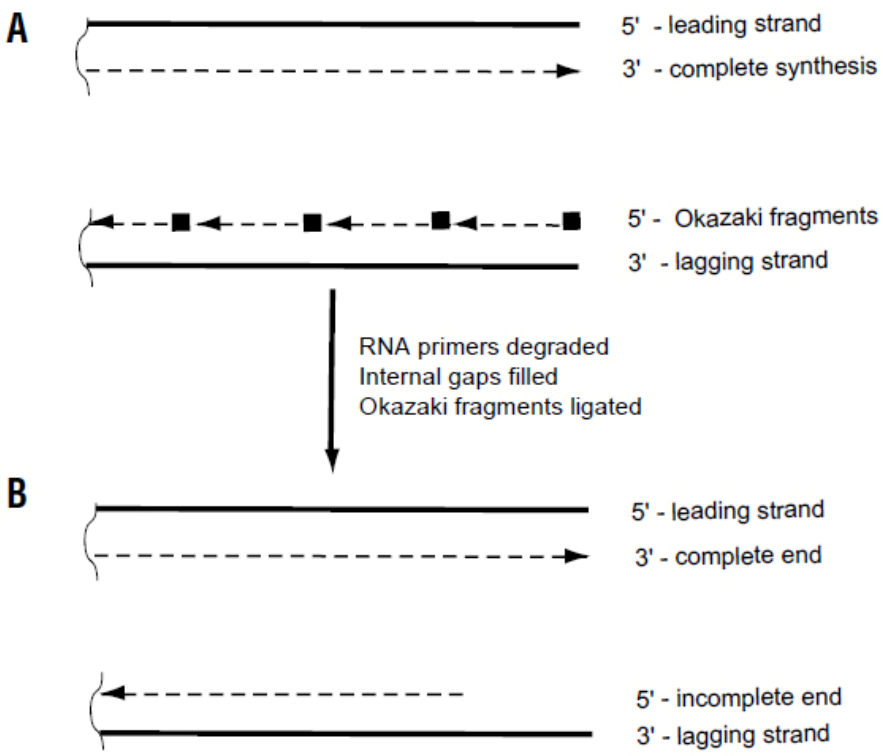
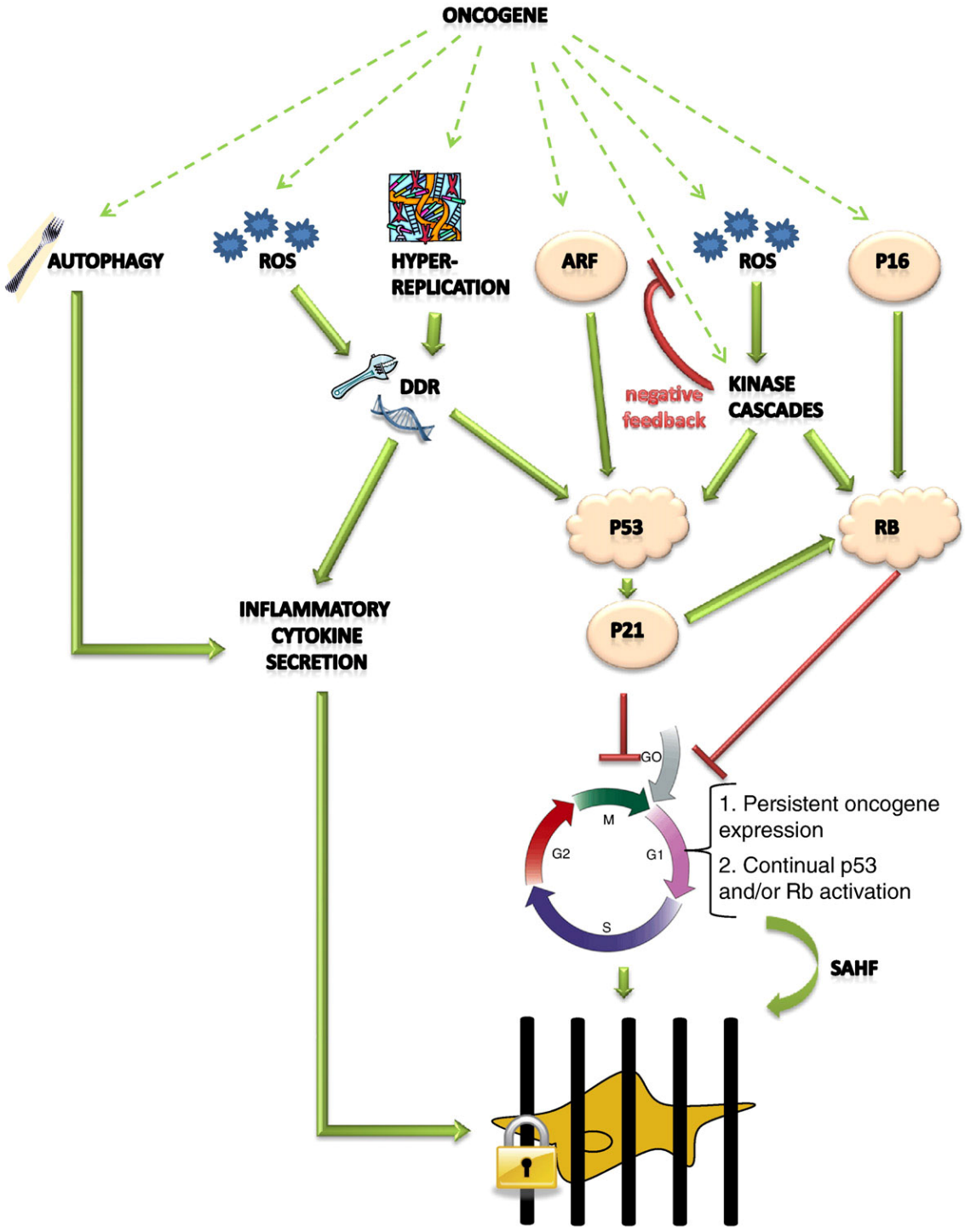

A
5' - leading strand
3' - complete synthesis
5' - Okazaki fragments
3' - lagging strand
RNA primers degraded
Internal gaps filled
Okazaki fragments ligated
B
5' - leading strand
3' - complete end
5' - incomplete end
3' - lagging strand

Figure 1-2 Hallmarks of Senescence.

Senescence induction activates multiple cellular processes whose goal is proliferation arrest. Betagalactosidase activity is not included in this diagram. Also Reactive Oxygen Species (ROS) was not discussed in this thesis. Diagram from McDuff and Turner[9]


ONCOGENE
AUTOPHAGY
ROS
HYPER-
REPLICATION
ARF
ROS
P16
DDR
negative
feedback
KINASE
CASCADES
P53
RB
INFLAMMATORY
CYTOKINE
SECRETION
P21
GO
M
G2
G1
S
1. Persistent oncogene
expression
2. Continual p53
and/or Rb activation
SAHF

Chapter 2

hTERT immortalization and regulation of intrinsic tumor suppressive barriers

As mentioned in Chapter I, telomere maintenance, mediated by expression of telomerase, is critical for evasion of proliferative senescence, a known barrier against cancer. Whether telomerase, in addition to providing cellular immortality, deregulates activation of intrinsic tumor-suppressing programs has been in debate[24, 53]. In this thesis we present evidence that expression of telomerase does not eradicate intrinsic tumor suppressive mechanisms.

Results

Distinct growth arrest programs in primary HMECs and HMEC/hTERT cells when expressing oncogenic Ras.

Expression of oncogenic H-Ras in murine and human cells triggers OIS, an irreversible cell cycle arrest [19, 24]. To determine whether ectopic expression of hTERT in human epithelial cells would rescue cells from this proliferative barrier we decided to study two parameters: DNA synthesis and the status of the Cell Cycle Machinery. In order to do this we transfected young primary HMECs and hTERT-immortalized HMECs with a plasmid containing the Sleeping Beauty dsRED transposon system containing H-RasV12 or empty vector as a control. Cells were pulsed overnight with Edu (a thymidine analog that identifies cells actively synthesizing DNA) and the proportion of Edu+ cells was analyzed. As expected primary HMECs expressing oncogenic RasV12 had a reduced proportion of Edu+ cells as compared to vector control. However, HMEC/hTERT cells expressing Rasv12 had increased proportion of Edu+ cells as

compared with vector suggesting that these cells were transiting into the cell cycle as shown in **Figure 2-1-A**

We also studied the status of cell cycle genes by western blotting, in particular genes involved in the Restriction Point. As expected, in primary HMECs oncogenic Ras induced a net suppression of cell cycle transit where phosphorylation of Retinoblastoma gene (Rb), guardian of the restriction point, was suppressed. However, in HMEC/hTERTs oncogenic Ras induced phosphorylation of Rb, an important commitment for transiting into the cell cycle as shown in **Figure 2-1-B**

The results presented above suggested that hTERT-immortalized cells had the capacity to overcome cell cycle arrest when expressing oncogenic Ras. We decided to study whether RasV12-expressing HMEC/hTERT cells were able to reach cell division. Using time-lapsed microscopy, we traced cells expressing RasV12 over a period of 2.5 weeks. To our surprise, RasV12 expressing cells were growth arrested suggesting that hTERT-immortalized cells induce post-replication arrest as shown in **Figure 2-1 C.**

HMEC/hTERT cells activate distinct tumor suppressive mechanisms when expressing oncogenic Ras compared to primary HMECs

Since HMEC/hTERT cells induced post-replicative arrest when expressing oncogenic Ras, we speculated that these cells might be inducing OIS. Several markers have been used for the identification of OIS as discussed above (see Hallmarks of Senescence section, Chapter 1). SA-β galactosidase has become the gold standard to screen whether certain conditions induce senescence. As expected in primary HMECs oncogenic Ras-induced β-galactosidase activity as seen by the blue/green histochemical staining[3424]. On the contrary, Ras-expressing HMEC/hTERT cells lacked β galactosidase activity **Figure 2-2.**

In primary human and murine cells oncogenic Ras induces deregulated DNA synthesis which sensitizes genomic DNA to single/double strand breaks and eventually arrests cell cycle transit (See DDF section in Chapter 1). Phosphorylation of histone H2A.X has become a characteristic marker for detecting DDR induction which can be identified as nuclear foci called DDF. In primary HMECs, oncogenic Ras induced a moderate DDR manifesting a lower number of DDF per nucleus. Meanwhile, HMEC/hTERT cells expressing oncogenic Ras had a robust induction of DDF by manifesting a shift into higher amounts of DDF per nucleus as shown in **Figure 2-3 A**.

It is also known that oncogenic Ras-induced OIS establishes changes in chromatin structure and organization which affect the expression of genes important for proliferation (See SAHFsection in Chapter 1). Methylation of Histone 3 on Lysine 9 has been considered a marker for detecting changes in chromatin condensation identified as nuclear foci called SAHF. Expression of oncogenic Ras in primary HMECs induced a robust formation of SAHF as observed in a shift for higher amounts of nuclear foci per nucleus. However, expression of oncogenic Ras established no changes in basal levels of chromatin condensation as shown in **Figure 2-3B.**

Persistent DDR signaling, caused by the accumulation of unrepaired lesions, can induce cell death by apoptosis (see Oncogene-Induced Senescence section, Chapter 1). Since, expression of oncogenic Ras in HMEC/hTERT cells induces a robust increase in γ H2AX, we decided to study whether apoptosis was triggered by these conditions. Initial experiments using flow cytometry showed that oncogenic Ras HMEC/hTERT cells had an increase in the sub G1 fraction compared to vector control. However when we assessed for Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), a marker for detecting cells in late stage apoptosis, there was no significant difference

compared to vector control. This result suggested that apoptosis was not the mechanism responsible for this increase in DNA fragmentation as shown in **Figure 2-3 C and D**.

Several studies have shown that besides triggering senescence, oncogenic Ras can lead to a caspase-independent cell death with features of autophagy[54, 55]. Autophagy is an evolutionarily conserved catabolic process activated in response to starvation or stress whereby cellular proteins, organelles and cytoplasm are engulfed in double-membrane vesicles, digested by fusion with lysosomes and recycled to sustain cellular metabolism [56]. Ultrastructural analysis showed an increased abundance of double and single membrane vacuoles in Ras-expressing HMEC/hTERT cells. Staining with vital acridine orange demonstrated that the vacuoles seen in Ras-expressing cells were acidic. Finally, staining for Lysosomal Associated Membrane Protein 1 (LAMP1) verified late stage autophagy in theses vacuoles as shown in **Figure 2-4 A-D**

Discussion

Telomere maintenance, mediated by expression of telomerase, is critical for evasion of proliferative senescence, a known barrier against cancer. Whether re-expression of telomerase, in addition to providing cellular immortality, deregulates activation of intrinsic tumor-suppressing programs has not been studied. We decided to study this question and hypothesized that breaching replicative senescence by ectopically expressing hTERT results in alterations that relax OIS and sensitize cells to Ras-induced neoplastic conversion. However, our data revealed that even after immortalization by ectopic expression of hTERT, cells activated distinct tumor suppressive mechanisms compared to those activated in Ras-expressing primary cells.

As expected, introduction of oncogenic Ras in primary HMECs induced a cell cycle arrest characteristic of OIS. However, to our surprise expression of Rasv12 in

HMEC/hTERT cells established an irreversible proliferation arrest at a post-DNA replication stage. These results support the contemporary concept that mammalian cells harness several intrinsic tumor suppressive mechanisms[1]. Based on these results we decided to determine which known mechanisms were activated to suppress the deregulated proliferation mediated by oncogenic Ras.

In primary fibroblasts, oncogenic Ras induces a wave of proliferation that promotes replication stress which causes DNA damage leading to apoptosis (if it is not repairable) or senescence[16-18]. It is also known that, oncogenic Ras establishes dramatic chromatin changes which consequently induce permanent cell cycle arrest [21]. Recent studies have described the interplay between DNA damage and heterochromatin condensation. At first oncogenes induce DDR, but later cells activate SAFH in order to suppress hyperactive DDR signaling and preserve viability of the cell.

Our results show that contrary to primary HMECs, expression of RasV12 in HMEC/hTERT cells induces a robust and persistent DNA Damage Response, but minimal chromatin condensation. In addition, this robust DNA damage is associated with substantial DNA degradation. Our results are in accordance with previous studies which have provided evidence that hTERT also regulates chromatin state and DNA damage responses[57]. However, the mechanism by which hTERT regulates these processes is still unknown.

DNA Damage is considered a tumor suppressive mechanism where the fate of the cell is either apoptosis or senescence. In our case, the observed increased DNA damage in hTERT cells might be working as a tumor surveillance mechanism. However increased DNA Damage sensitizes the cell for genomic instability. This step, on the other hand, promotes accumulation of mutations and other types of genetic lesions that favor

evolution of cancer. Although we observe substantial cell death in Ras –espressing hTERT, we cannot rule out that a small proportion would benefit from genomic instability and be the initiators of cancer.

While Ras-expressing HMEC/hTERT cells had a substantial amount of DNA degradation, we could not find evidence of induction of apoptosis by TUNEL assay. However, our results show a robust induction of autophagy with features of nuclear destruction. A recent study by Elgendy and others presented evidence that oncogenic Ras can induce destructive autophagy as a tumor suppressive mechanism. Moreover, this study presents evidence of a partial signaling circuitry responsible for the induction of this non-canonical activation of autophagy. Future studies in our system could elucidate whether autophagy is responsible for the observed cell death.

.

Materials and Methods

Cell Culture: Primary Human Mammary Epithelial Cells immortalized with human telomerase (HMEC/hTERT) were kindly provided by Dr Robert Weinberg's lab. Cell lines are maintained in mammary epithelium basal medium (MEBM, Lonza) supplemented with 10 ng/mL human epidermal growth factor (EGF), 5 ug/mL insulin, 0.5 ug/mL hydrocortisone, 52 ug/mL bovine pituitary extract, 50 ug/mL gentamicin, and 50 ng/mL amphotericin-B (Lonza), plus 10^{-5} mol/L isoproterenol and 5 ug/mL transferrin.

Western Blot Analysis: cells are lysed using Ripa buffer protease/phosphatase inhibitor cocktail, and equal amounts of cell extract protein per lane were subjected to SDS-PAGE, and blotted onto nitrocellulose membranes. For autophagy marker, Beclin 1 (Rabbit polyclonal antibody, 1:1000, BD Pharmingen). For cell cycle machinery, antibodies directed against Cyclin D1 (mouse polyclonal antibody, 1:500, BD Pharmingen), CDK 4 (mouse polyclonal antibody, 1:1000, Cell Signaling), p16 (mouse polyclonal antibody, 1:1000, BD Pharmingen), p21 (mouse antibody, 1:500; Cell Signaling), Rb detection kit (Cell Signalling). GAPDH immunoblotting (rabbite polyclonal antibody, 1:1000, Santa Cruz) was used as a loading control.

DNA synthesis analysis Cells were pulsed for 16 hours with Edu, fixed with Formalin and used protocols as described by the company (Invitrogen).

Vector and RasV12 constructs: The Vector construct is a transposon containing CMV promoter with an intron and SV40 polyA constructed by Dr. Scott McIvor. To this construct, H-RasV12 was subcloned from a purchased plasmid (Clontech). The vector and RasV12 constructs were analyzed by sequencing, and functionally tested by transfection into cells for red fluorescence.

Beta-Gal and Acridine Orange analysis: Beta-Gal assay was performed using the kit purchased from Cell Signaling following the manufacturer's protocol. For Acridine Orange, cells were stained with Acridine Orange 1% solution (Invitrogen) for 15 min in a 37°C, 2% CO_2 incubator. Acridine Orange was removed and the cells were washed once with HBSS. Phase-contrast and fluorescent images of the live cells were immediately taken.

Immunocytochemistry for γH2AX and H3 Lysine 9 Thrimethylated. Cells were plated in coverslips, transfected with RasV12 and Vector constructs. 48 hours post transfection, cells werewashed with PBS, fixed for 15 min at 25°C in 3.7% paraformaldehyde, permeabilized with 0.5% Triton solution in PBS for 1 hour at room temperature. Then cells were blocked with with 5% Goat serum solution for 30 minutes. Cells were immunolabeled with monoclonal anti-phospho-H2AX (Ser139) or monoclonal anti Trimethyl –Histone H3 (Lys (9) antibodies (Millipore), followed by Alexa 488-tagged secondary antibody (Invitrogen). Cells were rinsed, dried and mounted under cover slips using Vectashield mounting medium containing DAPI (Vector Laboratories, Inc.). Cells were observed at 60X magnification using a Bio-Rad MRC 1024 Multi-Photon Confocal microscope.

Immunohistochemistry for LAMP1. Briefly, Cells were plated at 60% confluence on glass 8 well chamber slides and transfected with RasV12 and Vector constructs. 48 hours post transfection, cells werewashed with PBS, fixed for 30 min at 37°C in 3.7% paraformaldehyde, permeabilized with Methanol:Acetone (1:1) solution for 10 minutes at -20°C. Then cells were blocked with 5% Goat serum solution for 30 minutes. Cells were immunolabeled with polyclonal anti-LAMP1 primary antibody (Affinity Bioreagents), followed by Alexa 488-tagged secondary antibody (Invitrogen). Cells were rinsed, dried and mounted under cover slips using Vectashield mounting

medium containing DAPI (Vector Laboratories, Inc.). Cells were observed at 60X magnification using a Bio-Rad MRC 1024 Multi-Photon Confocal microscope.

Betagalactosidase Assay We follow instructions as depicted by the manufacturer (Cell Signaling).

TUNEL Assay We follow instructions as depicted by the manufacturer (Millipore).

Figure 2-1 Oncogenic Ras-expressing HMEC/hTERT cells arrest at a post replicative stage as compared to primay HMECs.

A) DNA incorporation. Six days post-infection, cells were pulsed with Edu overnight and prepared for staining. B) Westernbloting for Cell cycle genes. C) Time lapse microscopy.

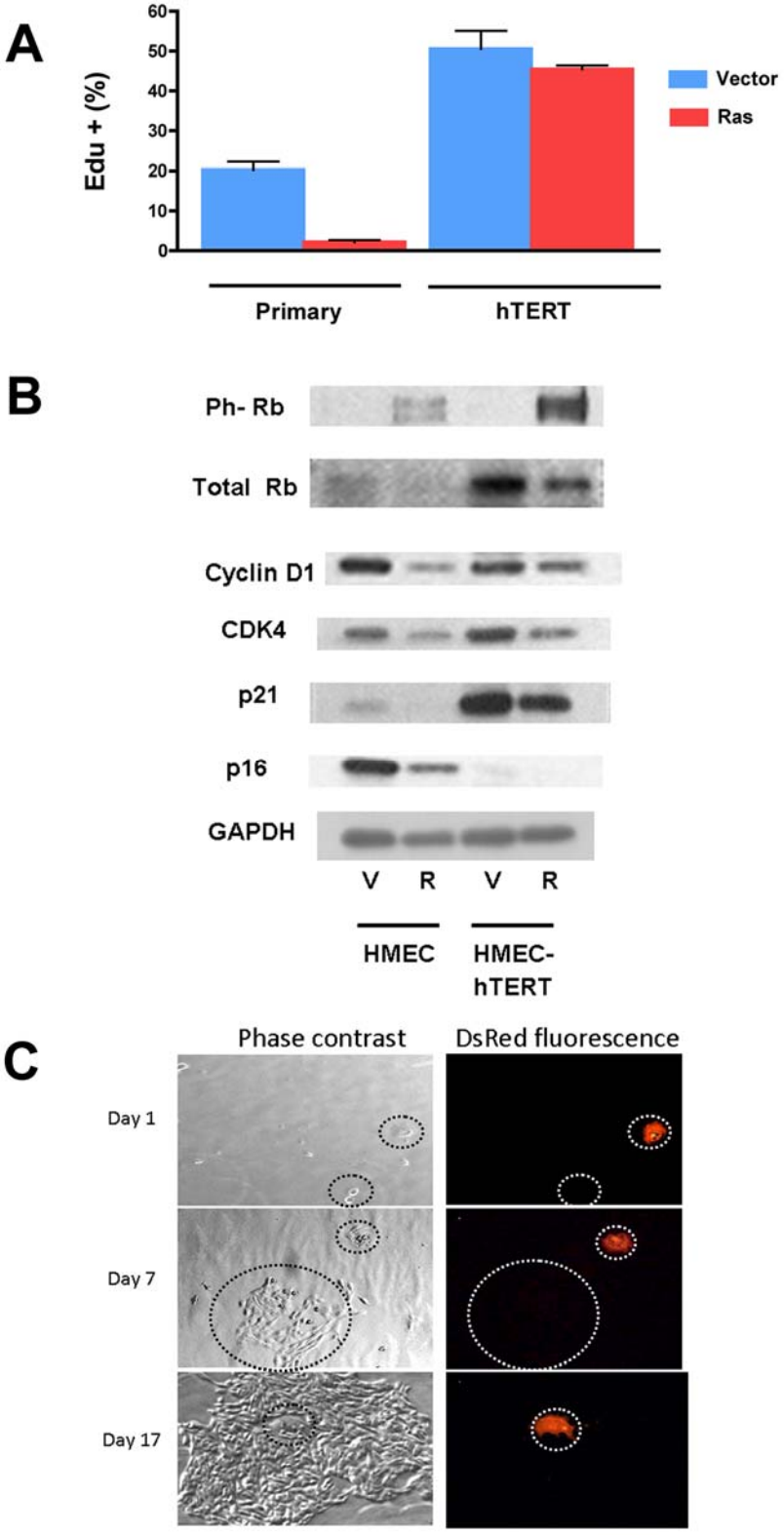

A
Edu + (%)
60
50
40
30
20
10
0
Vector
Ras
Primary
hTERT
B
Ph- Rb
Total Rb
Cyclin D1
CDK4
p21
p16
GAPDH
V R V R
HMEC
HMEC-hTERT
C
Phase contrast
DsRed fluorescence
Day 1
Day 7
Day 17

Figure 2-2 Immortalized cells do not manifest Beta galactosidase activity.

Primary HMECs and hTERT immortalized HMECs were transiently transfected with dsRED vector/RasV12 containing plasmid. 48 hours post transfection, cells were fixed and stained for Betagalactosidase activity following protocols recommended by the provider (Cell Signaling)

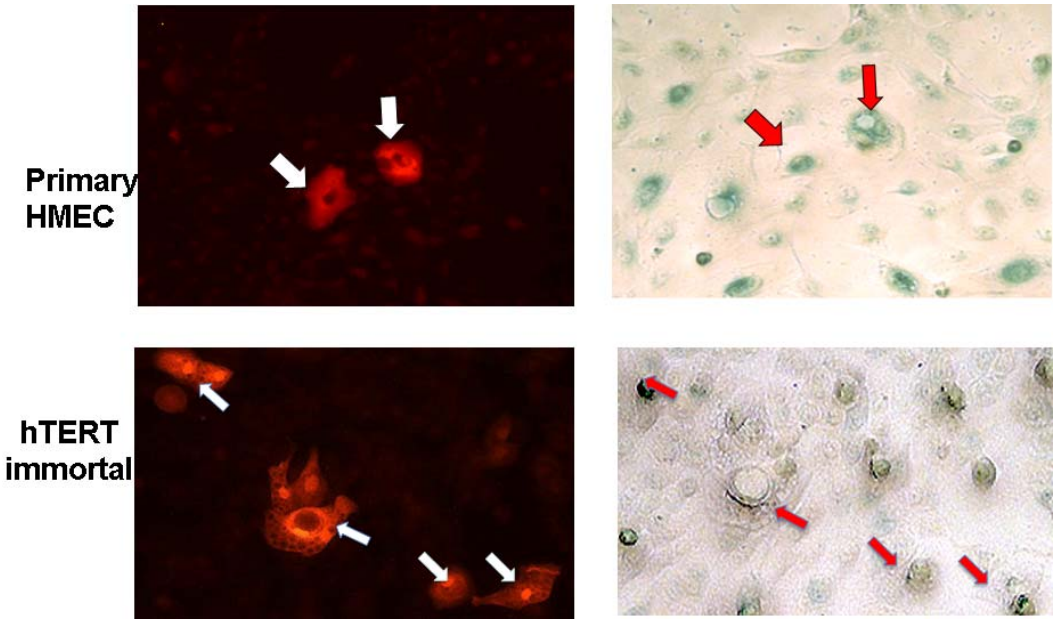

Primary
HMEC
hTERT
immortal

Figure 2-3 hTERT-immortalized cells induce different tumor suppressive mechanisms against oncogenic Ras.

A) Immunocytochemistry against γH2AX. B) Immunocytochemistry against H3 Tri-methyl Lys 9. C) Sub G1 fraction by flow cytometry. D) TUNEL Assay

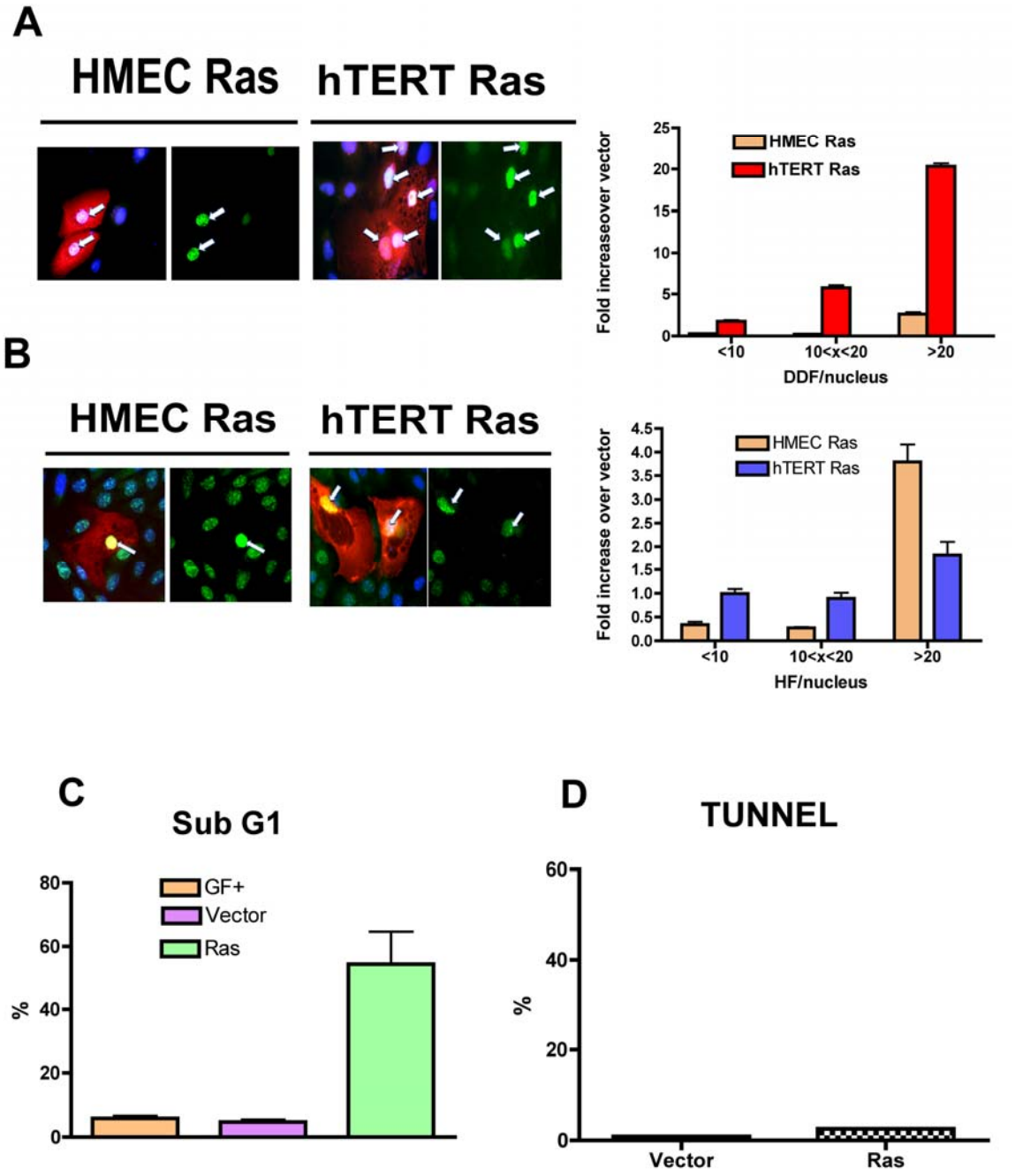
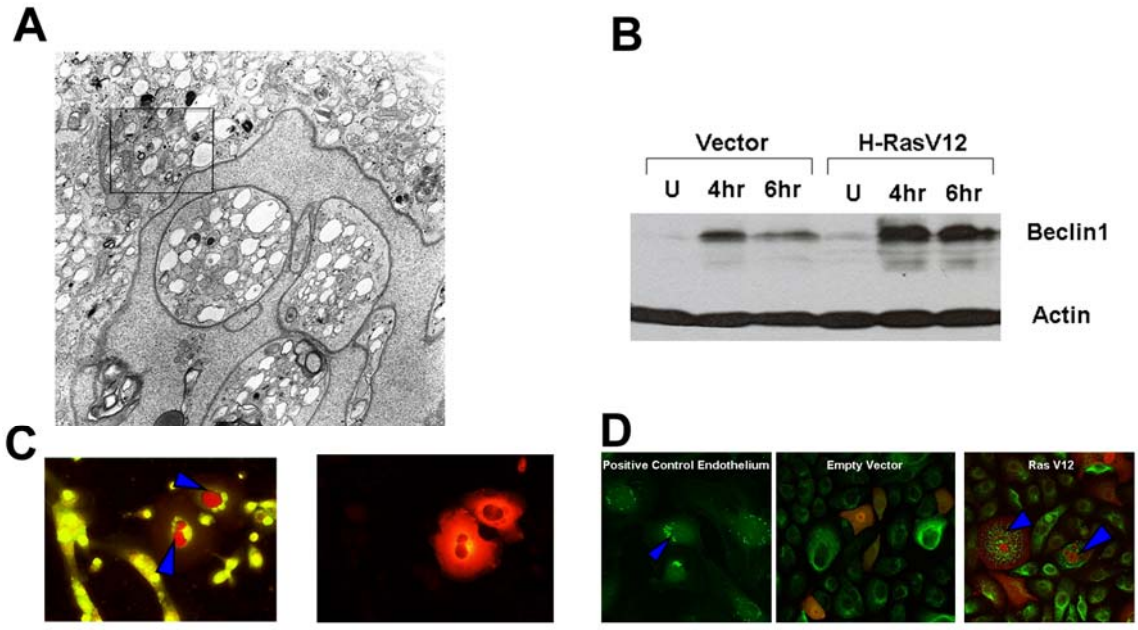

A
HMEC Ras
hTERT Ras
Fold increaseover vector
HMEC Ras
hTERT Ras
<10
10<x<20
>20
DDF/nucleus
B
HMEC Ras
hTERT Ras
Fold increase over vector
HMEC Ras
hTERT Ras
<10
10<x<20
>20
HF/nucleus
C
Sub G1
GF+
Vector
Ras
%
D
TUNNEL
%
Vector
Ras

Figure 2-4 hTERT-immortalized cells induce destructive autophagy when expressing oncogenic Ras.

A) Transmission electron microscopy. Notice presence of vesicles in nucleus. B) Western blotting for Beclin 1. C) Staining of acridine orange (red) colocalizes with vacuoles in Ras expressing cells. D) Immunofluorescence for LAMP1 marker.


A
B
Vector
H-RasV12
U
4hr
6hr
U
4hr
6hr
Beclin1
Actin
C
D
Positive Control Endothelium
Empty Vector
Ras V12

Chapter 3

Controversial views regarding the role of immortalization in breaching oncogene induced senescence barriers

Currently, there has been a big debate on whether or not immortalization mediated by hTERT inhibits Ras-induced oncogene induced senescence. On the one hand several studies using primary epithelial and fibroblast cells, have provided evidence that hTERT does not rescue cells from OIS and therefore other genetic alterations (such as suppression of the Rb/p16 axis) are needed to overcome this irreversible arrest[24, 44, 45]. Recent studies, however, have provided evidence that immortalization mediated by ectopic expression of hTERT while promoting accumulation of some senescence-associated markers, fails to trigger permanent growth arrest [51, 53].Therefore, hTERT does rescue cells from OIS.

Conflicting results

In the previous chapter, we presented evidence that ectopic expression of hTERT did not suppress activation of tumor suppressive mechanisms. Moreover, our results showed a distinct tumor suppressive program activated by hTERT immortalized cells to suppress deregulated growth by oncogenic Ras. Therefore, our results seemed to support the argument that hTERT cannot overcome OIS.

To further characterize the durability of Ras-induced cell proliferation arrest, we decided to study the kinetics of this process in mass culture by stably transfecting HMEC/hTERT cells with the Sleeping Beauty dsRED transposon containing H-RasV12 or empty vector

as a control. Although cells expressing dsRED were FACS sorted, during the process of cultivation some cells lost dsRED expression. This situation offered us the opportunity to study changes in cell composition in a mixed population of dsRED positive and negative cells. Cells were collected at different time points and the percentage of DsRED positive cells was determined by flow cytometry as shown in **Figure 3-1 A**

As previously observed in time lapse experiments, the proportion of red cells expressing oncogenic Ras decreased over time. To our surprise in certain period of time the proportion of oncogenic Ras cells changed, switching to a slowly growing expansion state as shown in **Figure 3-1 B.**

Conclusion

The results mentioned above suggested that hTERT-immortalized cells mount different responses to oncogenic Ras. At the beginning, the population of cells expressing oncogenic Ras underwent proliferation arrest. However, eventually a population of cells overcame this proliferation block and resumed the Ras–induced proliferative program. This result contrasts with the response that primary cells undergo once they express oncogenic Ras: a homogeneous irreversible proliferation arrest.

Hypothesis: Heterogeneity of HMEC/hTERT cells

To explain the observed diversity in outcomes of oncogenic Ras in HMEC/hTERT cells, we considered the possibility that the HMEC/hTERT population is heterogeneous; and the differential response to oncogenic Ras results from a durable property acquired by the cells as a result of their liberation from the constraints of replicative senescence (or other properties conferred by hTERT). Alternatively, the response to oncogenic Ras might reflect the normal distribution of any uniform cell population to a perturbation (i.e. stochastic events).

Results

In order to test this hypothesis, we isolated 8 randomly selected clonal cell lines from the HMEC/hTERT cell population and challenged each of the clonal lines with oncogenic Ras. We reasoned that if the HMEC/hTERT cell population was heterogeneous, then we expected clonal lines to either manifest OIS or not. Using DNA synthesis as the primary readout, the following results supported our intention: a) 1 clonal line that had arrested DNA synthesis after introduction of oncogenic Ras, b) 3 clonal lines that showed a distribution of different levels of DNA synthesis, c) and 1 line in which Ras prompts robust DNA synthesis as shown in **Figure 3-2** .

Conclusion

Our results provide evidence that the population of HMEC/hTERT cells is heterogeneous and, therefore, diverse responses to oncogenic Ras manifest. In this case we can observe that the majority of cells permanently arrest while a small group of cells spontaneously resumed cell proliferation.

Characteristics of isolated clones when expressing RasV12

As mentioned above while many cells induced proliferation arrest, some cells were able to escape from this barrier. This suggested that cells might also have other different characteristics besides cell proliferation. For this purpose, we selected two clones that had different proliferation capacities when expressing oncogenic Ras (Clone 7, high proliferation; Clone 5, low proliferation), and asked whether these cells exhibit cancer associated properties.

Results

Based on their differences in DNA synthesis capacity we speculated that the clones might induce DDR axis and chromatin condensation differently. In clone 5, oncogenic

Ras induced a moderate DDR manifesting a lower number of DDF per nucleus. Meanwhile, in clone 7 expression of oncogenic Ras resulted in a robust induction of DNA damage by manifesting a shift into higher amounts of DDF per nucleus. In addition, results from flow cytometry analysis showed that oncogenic Ras induced strong DNA degradation in clone 7 as shown by a robust amount in the sub G1 fraction compared to clone 5. Finally, no differences were noticed between clones in terms of heterochromatin condensation as shown in **Figure 3-3 A-C.**

To determine whether clones had potential to form tumors, we decided to test for colony forming capacity and anchorage independent growth. When plated at low density, Ras-expressing clone 5 cells did not form colonies during 5 days of incubation in plastic. Moreover, when cells were plated in soft agar, all cells had characteristic apoptotic morphology after 48 hours post seeding. Alternatively, only clone 7-oncogenic Ras cells efficiently formed colonies on plastic, and grew in soft agar in a glandular pattern that profoundly differed from the large, amorphous multilayer spheroids formed by breast carcinoma cells as shown in **Figure 3-4**.

Knowing that clone 5 and 7 had differences in the cancer associated properties mentioned above, we asked whether differences in Ras-downstream signaling pathways might exist. We chose detection of active MAPKinase and PI3K pathways because these circuitries are involved in proliferation and survival of the cell. Results by westernblotting showed that clone 7 had a strong activation of these pathways when expressing Rasv12 as compared to clone 5 as shown in **Figure 3-5.**

Discussion

Currently, there has been a big debate on whether immortalization mediated by hTERT is able to breach intrinsic barriers against oncogene-mediated transformation. However,

results from this study help to reconcile the controversial observations made by different laboratories by incorporating the conceptual structure of heterogeneity in a population. Our data suggests that the process of immortalization promotes heterogeneity in the activation of tumor suppressive mechanisms in the population, and therefore, diverse responses to oncogenic Ras manifest. These results contrast with the response that primary cells undergo once they express oncogenic Ras: a homogeneous irreversible proliferation arrest

In Chapter II we provided evidence supporting the idea that immortalization by hTERT did not suppress activation of tumor suppressive mechanisms. To our surprise experiments studying temporal analysis of a composition of cells provided an unexpected result: some cells were able to breach proliferation arrest. To explain these conflicting results, we hypothesized that the population of HMEC/hTERTcells were heterogeneous. The isolation of several clones and the finding that differences in proliferation capacity existed between them supported our hypothesis.

We further asked whether this heterogeneity in culture fostered gain of cancer-associated properties. As predicted one of the clones had robust colony forming capacity as well as anchorage independent growth while the other clone succumbed to the intact tumor suppressive barrier. Moreover, differences in Ras-downstream signaling were noticeable between these clones. These results further support the argument of heterogeneity mediated by immortalization.

We also found differences in the activation of DDR between clones. DNA Damage is considered a tumor suppressive mechanism. However, persistent DNA Damage sensitizes the cell for genomic instability and accumulation of genetic lesions which could favor the evolution of cancer[20]. Whether deregulated DDR is the mechanism that,

in hTERT immortalized cells, promote gain of cancer associated properties remains to be determined.

In our system, immortalization of HMEC cells was mediated by exogenous expression of hTERT. The discovery of hTERT as a reverse transcriptase clarified the mechanism by which eukaryotic cells replenished their telomeres and consequently avoided senescence. However, current studies suggest that hTERT supports proliferation and survival of cells through mechanisms that are independent of its telomere-elongation function[58, 59]. Whether these alternative functions of hTERT are responsible for the heterogeneity of the population would be for future directions.

Our results provide evidence that immortalization by hTERT mediates heterogeneity in culture. An interesting question that follows is whether cells depend on hTERT expression to maintain culture heterogeneity. Currently we have isolated clones with an shRNA against human hTERT and study whether loss of hTERT function renders cells susceptible to re-activation of tumor suppressive mechanisms. However, it might be possible that accumulated genetic/epigenetic alterations have changed the regulation of anti cancer barriers rendering cells insensitive to the absence of hTERT. Finally because these cells have been expressing hTERT for a vast amount of time, cells might use other mechanism s to avoid a limited life span (an example of which could befor example ALT).

In summary, our main finding consists with the idea that breaching the replicative senescence barrier by hTERT – a frequent event in naturally occurring malignancies – leads to a cell population in which some cells spontaneously have lost the ability to activate OIS and are therefore susceptible to neoplastic conversion by oncogenic Ras. This study provides a new perspective on the role of hTERT in carcinogenesis: hTERT

provides a framework for relaxation (likely genetic or epigenetic alterations) of intrinsic barriers that prevent cancer development.

Material and Methods

DNA synthesis analysis Cells were pulsed for 16 hours with Edu, fixed with Formalin and used protocols as described by the company (Invitrogen).

Western Blot Analysis: Cells were lysed using RIPA buffer containing, protease/phosphatase inhibitor cocktail, and equal amounts of cell extract protein per lane were subjected to 10% SDS-PAGE, blotted onto nitrocellulose membranes, and identified using the following antibodies: phospho p44/442 and total p44/42 (Cell Signaling), phospho and total Akt (Cell Signaling) and Actin (Sigma) used as a loading control.

Immunocytochemistry for γH2AX and H3 Lysine 9 Thrimethylated. Cells were plated in coverslips, transfected with RasV12 and Vector constructs. 48 hours post transfection, cells werewashed with PBS, fixed for 15 min at 25°C in 3.7% paraformaldehyde, permeabilized with 0.5% Triton solution in PBS for 1 hour at room temperature. Then cells were blocked with with 5% Goat serum solution for 30 minutes. Cells were immunolabeled with monoclonal anti-phospho-H2AX (Ser139) or monoclonal anti Trimethyl –Histone H3 (Lys (9) antibodies (Millipore), followed by Alexa 488-tagged secondary antibody (Invitrogen). Cells were rinsed, dried and mounted under cover slips using Vectashield mounting medium containing DAPI (Vector Laboratories, Inc.). Cells were observed at 60X magnification using a Bio-Rad MRC 1024 Multi-Photon Confocal microscope.

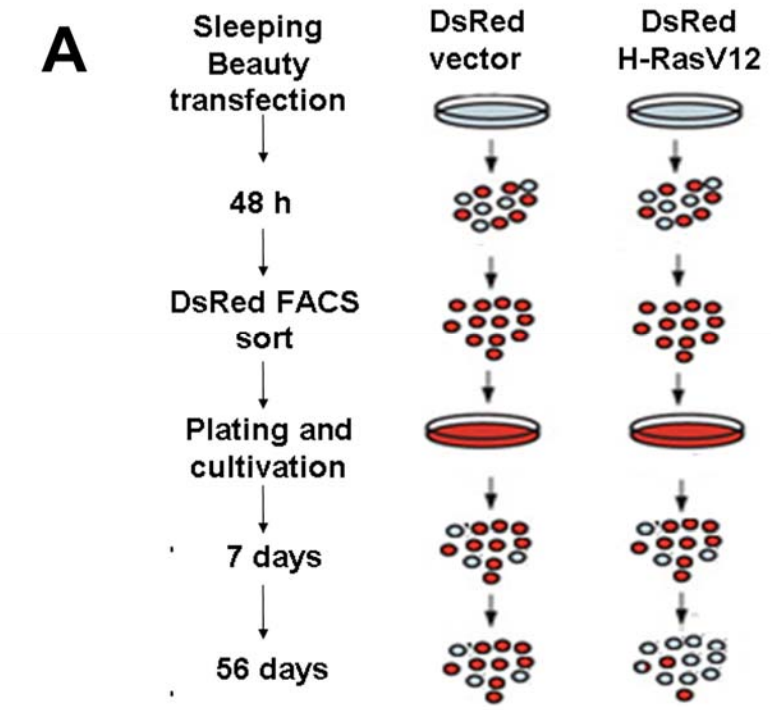
Anchorage-dependent colony formation: Cells were seeded into 6-well clusters at a density of 500 cells/well. Cultures were continued for 5 days, fixed with 4% formaldehyde, and stained with Coomassie Blue.

Anchorage-independent growth: Cells were seeded into soft agar at a density of 2 $X10^4$ cells/well in 6-well plates with a bottom layer of1%SeaPlaque agarose (BioWhittaker Molecular Application, Rockland, ME) and a top layer of 0.6% SeaPlaque agarose in MEBM supplement with growth factors and antibiotics. Growth patterns were analyzed by microscopic observation after 2 weeks.

Flow cytometry: For quantification of DNA content, cultured cells were detached with trypsin, washed with PBS, fixed with ice-cold 70% ethanol, and resuspended in propidium iodide (PI) staining mixture. The percentage of cells with sub G1 DNA content was determined on a FACSCalibur flow cytometer (Beckson Dickson). For quantification of proportion of dsRED cells in culture, cells were collected and resuspended in 1X PBS containing 1% growth Factors.

Figure 3-1 HTERT-Immortalized cells do not show a uniform response against oncogenic Ras.

A) Scheme of temporal analysis. B) Flow cytometry analysis during stipulated time course


A
Sleeping Beauty transfection
48 h
DsRed FACS sort
Plating and cultivation
7 days
56 days
DsRed vector
DsRed H-RasV12

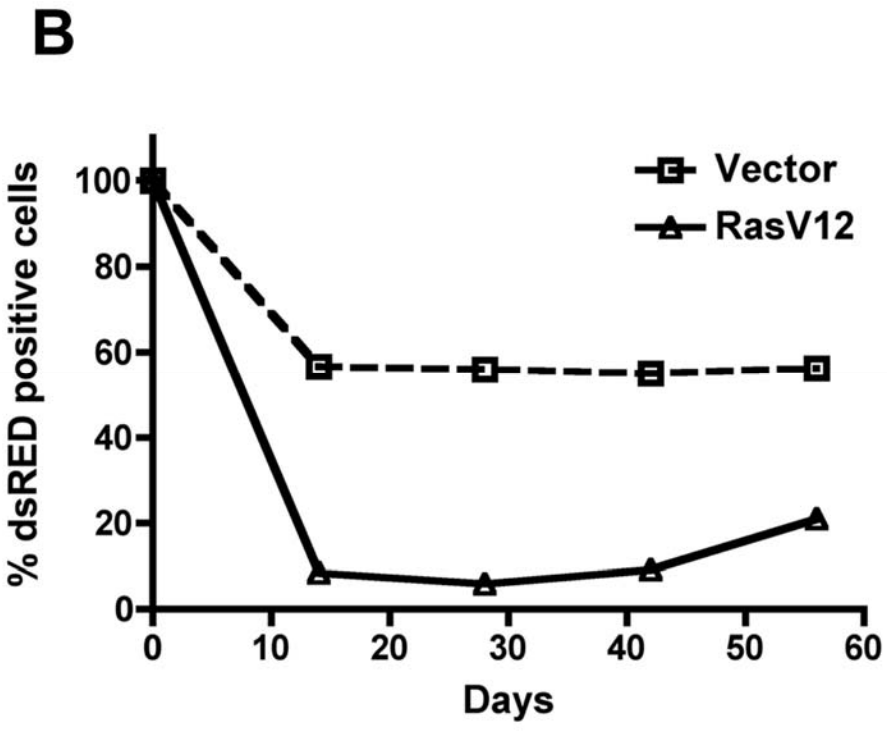

B
% dsRED positive cells
100
80
60
40
20
0
0
10
20
30
40
50
60
Days
Vector
RasV12

Figure 3-2 Clone 7 and clone 5 show different proliferation responses induced by oncogenic Ras.

In this picture two clones were selected as examples which have with diametrically opposite DNA synthesis capacity.

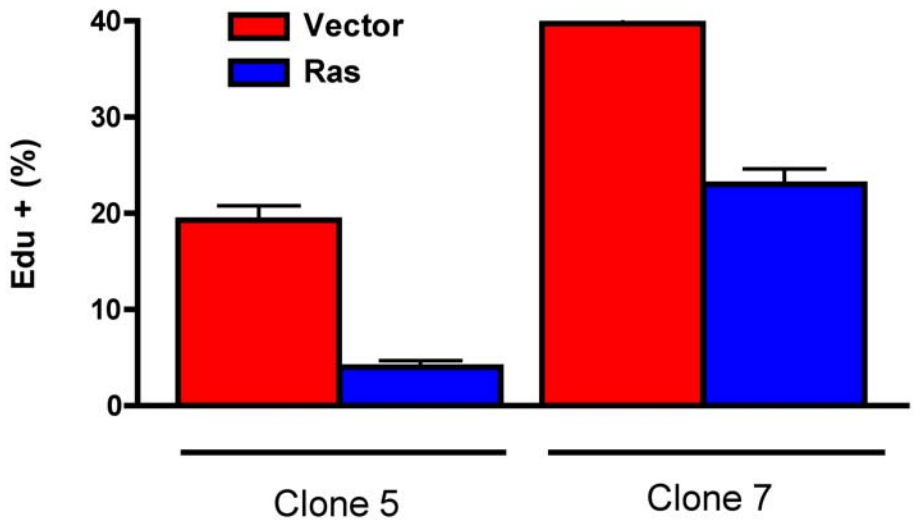

Vector
Ras
Edu + (%)
40
30
20
10
0
Clone 5
Clone 7

Figure 3-3 Characteristics of the isolated clones when expressing oncogenic Ras.

A) Quantification of γH2AX (DDF). B) Quantification of SAHF. C) SubG1 fraction quantified by flow cytometry

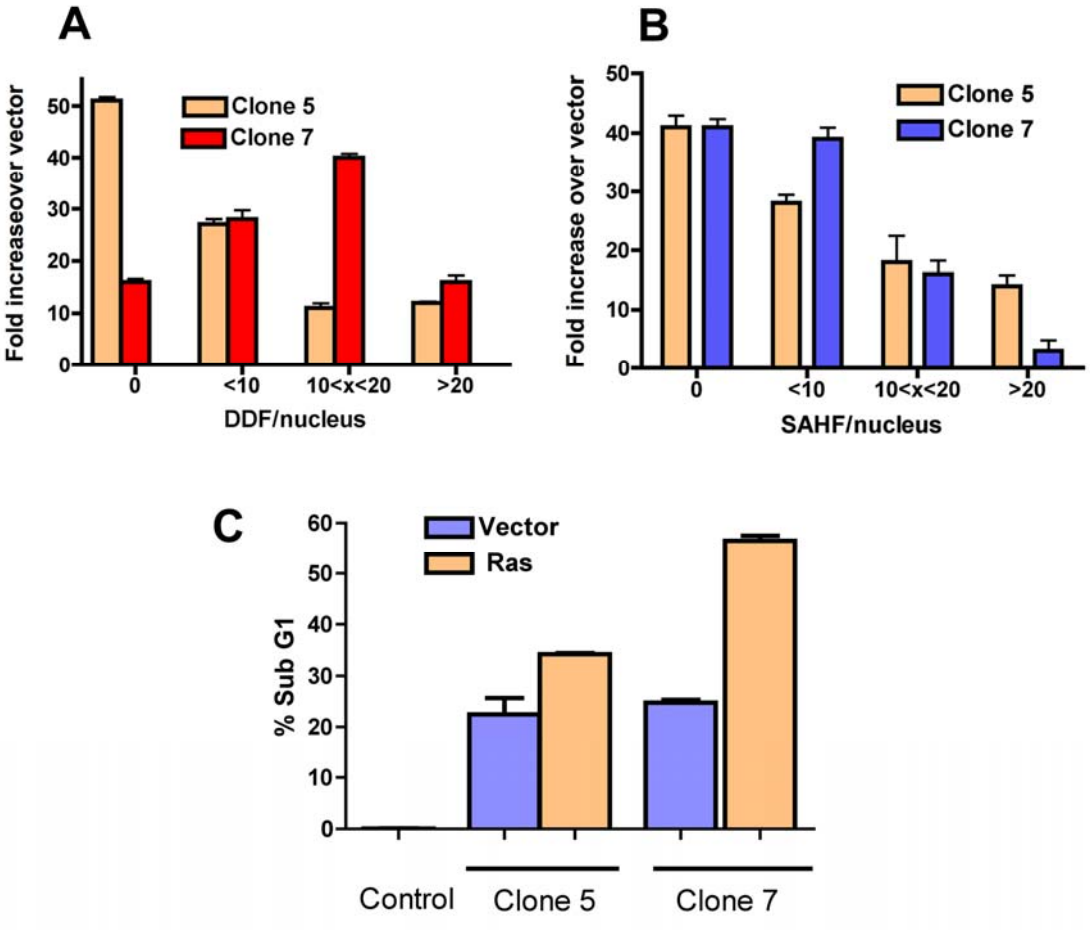
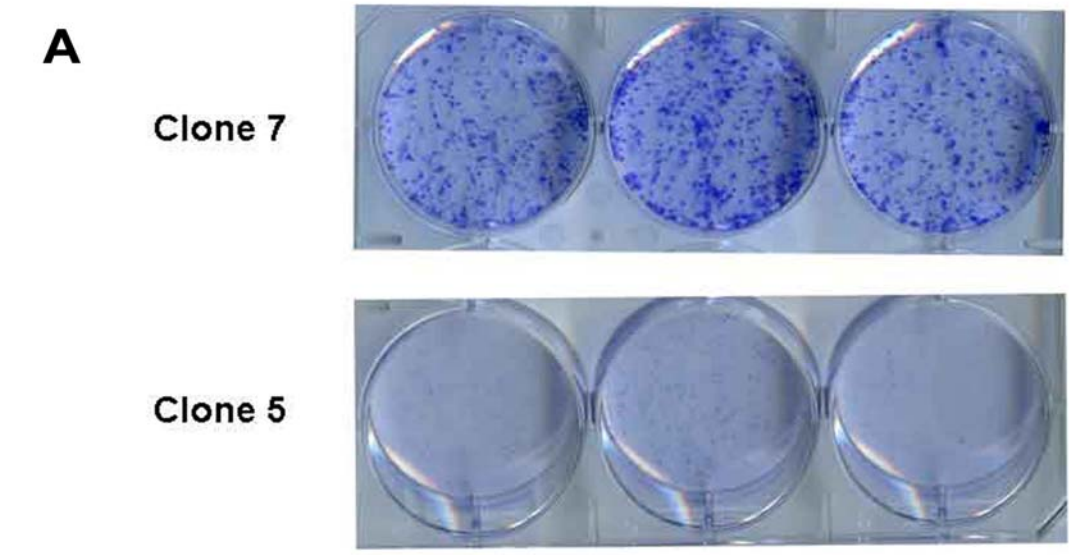

A
Fold increaseover vector
Clone 5
Clone 7
0
10
20
30
40
50
0
<10
10<x<20
>20
DDF/nucleus
B
Fold increase over vector
Clone 5
Clone 7
0
10
20
30
40
50
0
<10
10<x<20
>20
SAHF/nucleus
C
% Sub G1
Vector
Ras
0
10
20
30
40
50
60
Control
Clone 5
Clone 7

Figure 3-4 Clone 7 and clone 5 posses different cancer associated properties.

A) Clonogenic Assay (5 day post plating). B) Anchorage Independent Assay for two time points


A
Clone 7
Clone 5

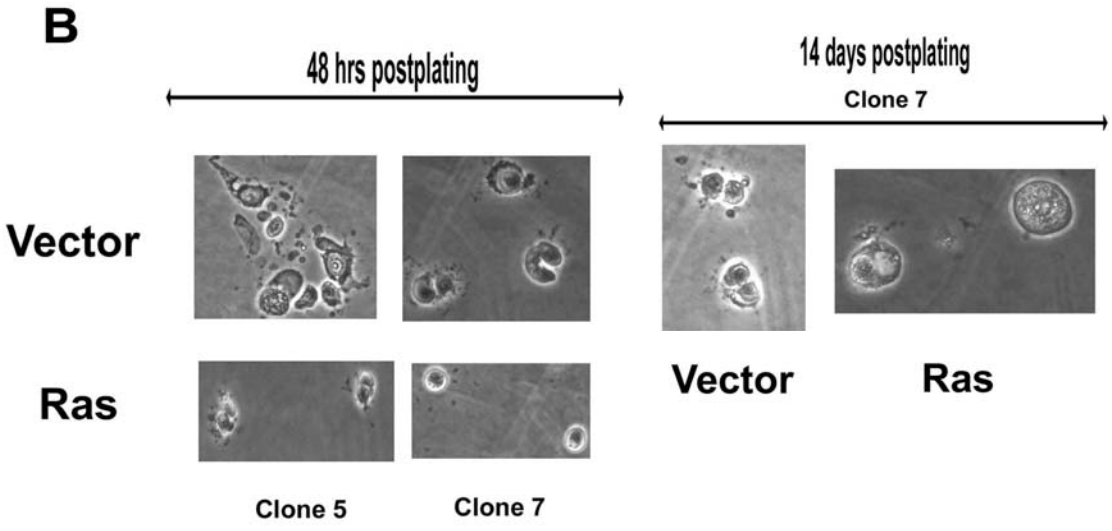
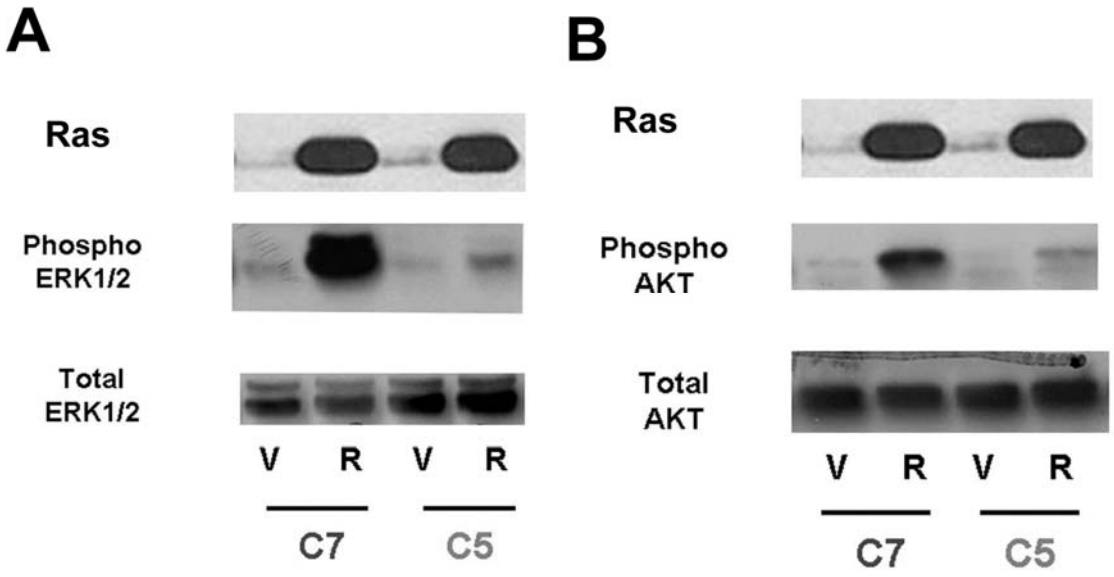

B
48 hrs postplating
14 days postplating
Clone 7
Vector
Ras
Vector
Ras
Clone 5
Clone 7

Figure 3-5 Isolated clones show differences in the activation of Ras-downstream signaling.

A) Western blotting for detection of activated MAPkinase. B) Western blot for detection of activated Akt.


A
Ras
Phospho ERK1/2
Total ERK1/2
V R V R
C7 C5
B
Ras
Phospho AKT
Total AKT
V R V R
C7 C5

Chapter 4 Thesis Conclusion

Currently, two known cellular processes have been designated as intrinsic tumor suppressive mechanisms. Apoptosis is a mechanism of cell death that is used by multicellular organisms to eliminate unwanted cells in a diversity of settings. Senescence, on the other hand, is an irreversible proliferation arrest that, similarly to apoptosis, is activated by different types of stimuli. In this thesis, senescence was the focus of our study.

Senescence was described more than 40 decades ago when Leonard Hayflick's work showed for the first time that normal human cells had a limited proliferation capacity in culture. Eventually, the discovery that maintenance of telomeric DNA was responsible for induction of senescence provided a mechanism that would explain how cells control and maintain homeostasis. This causal relationship was established when introduction of the hTERT gene provided indefinite proliferation to several primary cells. Therefore the relationship between reactivation of telomerase enzyme and unlimited proliferation capacity was established.

As mentioned previously telomere maintenance, mediated by expression of telomerase, is critical for evasion of proliferative senescence. However it has not been clear whether telomerase, in addition to providing cellular immortality, deregulates activation of intrinsic tumor-suppressing programs. Results from this thesis help reconcile the different observations made and can be summarized in two statements: 1) Immortalization by hTERT does not abrogate activation of intrinsic tumor suppressive mechanisms, but 2) promotes variability in the activation of these mechanisms providing a window of opportunity for the evolution of cancer.

We found that contrary to primary cells, hTERT immortalized cells respond differently to oncogenic Ras by undergoing post-replication arrest. Moreover, we found that these cells had increased DNA damage/degradation and destructive autophagy as parallel barriers to suppress cancer progression. Therefore, immortalization by hTERT does not eliminate activation of these processes. Whether these mechanisms manifest in hierarchical order to suppress different stages of transformation or not might be an idea to think about.

Although these barriers are available, the entire population of cells does not respond uniformly to oncogenic Ras. Instead, we observed variability in the execution of these responses. Our results suggest that the process of immortalization fosters the opportunity for evolution of the culture. In these conditions many cells can harness mechanisms of defense while a minority of cells loses the capacity to respond.

Based on the gathered data we propose the following model as shown in **Figure 4-1.** In this model, expression of hTERT, besides rescuing cells from replicative senescence, provides a framework for relaxation of intrinsic tumor suppressive barriers (genetic or epigenetic alterations). This process generates heterogeneity in the population where these mechanisms of defense might be intact or deregulated. This model predicts that in a population of cells while most capable of activating these barriers, some of the cells are insensitive to them and progress in their path to become tumors.

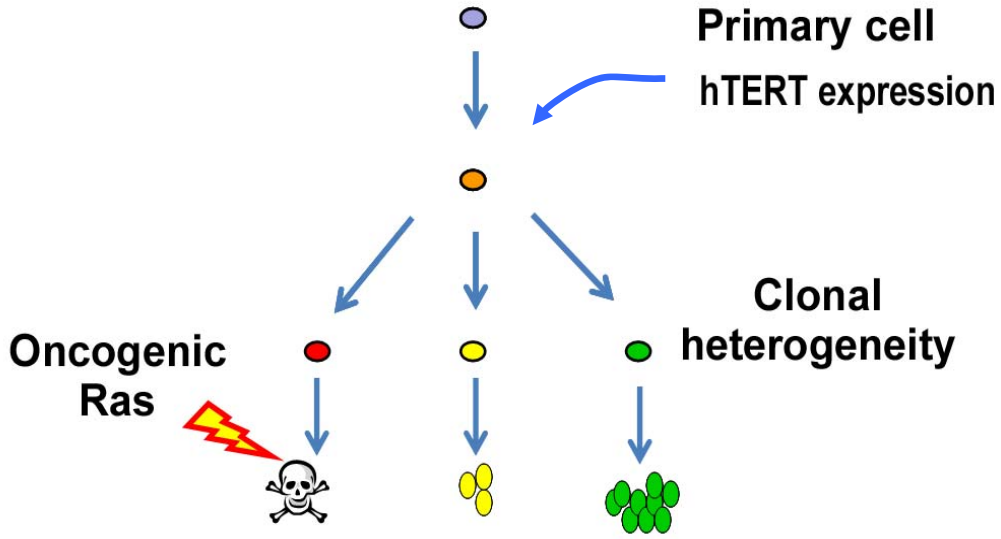
Currently, telomerase inhibitors have entered phase I/II clinical trials in several malignancies. Inhibition of telomerase, in theory, would promote telomere erosion and therefore cell crisis ending up in cell death. However recent studies suggest that these well-intended therapeutic strategies might not be a practical solution to control cancer.

Studies from DePinho's lab presents evidence that re-expression of telomerase in mice cells that have reached telomere attrition, supports malignancy thanks to genomic gains accumulated as a result of genomic instability. Nevertheless, inhibition of telomerase in tumors that are dependent on its reactivation is bypassed by activation of the alternative lengthening of telomeres (ALT) pathway [60, 61]. Although these results open a Pandora's Box, hope is not lost. The following quote from Peter Campbell provides light in this consternation: "with every therapeutic bypass, the clone makes sacrifices, and these become themselves rational therapeutic targets"[62].

In summary this thesis adds to the mounting evidence that mammalian cells possess intrinsic tumor suppressive mechanisms that are harnessed to suppress oncogene-mediated transformation. Therefore, transformation of cells relies on breaching these mechanisms. This body of work provides evidence that the process of immortalization promotes a framework for heterogeneity in a cell population which promotes future development of tumors.

Figure 4-1 Model of our thesis

hTERT enables heterogeneity in a population of HMEC which sustains oncogene sensitive and resistant clones


Primary cell
hTERT expression
Clonal heterogeneity
Oncogenic Ras

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