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14. ABSTRACT

Breast tumor cells display great diversity in gene and protein expression, and genomic alterations. Distinct cancer subtypes have been categorized, with striking differences in clinical parameters. Our approach to understanding the molecular pathways that lead to the different types of breast cancer in vivo has been to model this process in vitro. However, thus far, almost all in vitro transformed HMEC lines represent a limited subset of in vivo cancer phenotypes. We hypothesized that this could result from restrictive and stressful culture conditions that don't support in vitro growth of the in vivo target cells. Our proposal seeks to generate lines more reflective of breast cancer phenotypes by using our improved, less stressful methods for normal HMEC culture that we now show supports long-term growth of cells with phenotypes of luminal, basal, and progenitor lineages, and to directly examine the effects of stress on vulnerability to oncogenic transformation. In the past year, we have shown that different HMEC populations vary in susceptibility to c-myc mediated transformation, and are currently examining the mechanism for this result. We have also found differences in epigenetic markers among these populations. The generation of diverse transformed HMEC lines with defined genetic alterations may aid the identification of potential therapeutic treatments.

15. SUBJECT TERMS
human mammary epithelial cells, c-myc, oncogenic transformation, stasis

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INTRODUCTION:

Recent studies have shown that cells derived from breast tumors display great diversity in patterns of gene and protein expression, and genomic alterations. Based on gene expression profiling, multiple distinct cancer subtypes have been categorized; importantly, these subtypes show striking differences in clinical parameters. Understanding the pathways of molecular alterations that lead to the different types of breast cancer in vivo could facilitate design of clinical interventions in the carcinogenic progression. One approach to examining human breast carcinogenesis is to model this process in vitro, starting with normal HMEC and using oncogenic agents to transform the normal cells to cancer. However, thus far, almost all in vitro transformed HMEC lines represent a limited subset of the phenotypes observed in breast cancer cells in vivo. We hypothesized that the limited phenotypes of in vitro transformed HMEC could result from culture conditions that restrict proliferation of normal HMEC to mostly cells with a basal phenotype. Normal cultured HMEC proliferate for a variable number of population doublings (PD) before encountering a first senescence barrier, stasis, which is stress-associated, mediated by the retinoblastoma (Rb) pathway, correlated with increased levels of $p16^{INK4a}$, and telomere length independent. HMEC may overcome stasis by inactivation of the Rb pathway, and continue growth until encountering a second extremely stringent barrier due to telomere attrition (Brenner et al, 1998, Romanov et al. 2001, Garbe et al. 2007). HMEC grown in a serum-free medium reach stasis quickly, and can show spontaneous silencing of p16, giving rise to the p16(-) post-stasis HMEC (called post-selection) that are commercially available (Hammond et al, 1984, Stampfer, 1985). In vitro transformed lines derived from post-selection HMEC have shown a basal phenotype (Stampfer and Bartley, 1985, Perou et al., 2000). We now propose to generate HMEC lines more reflective of the in vivo spectrum of breast cancer phenotypes by using improved methods for growing normal pre-stasis HMEC. We previously defined low stress culture conditions that allow pre-stasis HMEC to grow for ~60 PD prior to p16 induction and growth arrest at stasis (Garbe et al., in prep). We have now shown that pre-stasis HMEC with phenotypes of luminal, basal, and progenitor lineages are present, indicating that these cultures represent *in vivo* populations more accurately than previous HMEC culture conditions. We have recently demonstrated that these different HMEC lineages can be isolated by flow sorting. We hypothesize that these heterogeneous, or lineage purified, unstressed pre-stasis populations will be more vulnerable to transformation when targeted by oncogenic agents, and may yield a greater range of transformed phenotypes. Our objectives include addressing the basic research questions - are unstressed pre-stasis HMEC more vulnerable to transformation and is a particular normal cell type more vulnerable or more likely to give a specific transformed phenotype, and producing a practical outcome – generation of useful transformed lines. Our approach will be: (1) Determine whether transduction of unstressed pre-stasis HMEC with oncogenes such as c-myc and Wnt-1, with or without p53 inactivation, will yield transformed lines with phenotypes representative of most human breast cancer cells, and if there are correlations of transformed cell phenotypes with the target cell population and/or agents used for in vitro transformation. (2) Experimentally examine whether cultured HMEC that have not encountered stress are more vulnerable to transformation than those exposed to stress. The generation of diverse transformed HMEC lines with defined genetic alterations may aid the identification of potential therapeutic treatments.

BODY:

To accomplish our ambitious goals, we have made collaborations with investigators at LBNL (Bissell and Gray labs), U. Arizona Cancer Center (Futscher lab) and Case Western Reserve U. (Jackson lab). For our first year, our lab has focused on questions relating to the effect of stress on vulnerability to transformation in different HMEC types.

Long-term growth of normal pre-stasis HMEC, and isolation of specific HMEC lineages. Our previous studies defined culture conditions that allow active, rapid long-term growth (up to ~60 PD) of normal reduction-mammoplasty, or milk-derived HMEC (Figure 1) (Garbe et al. in prep). We have now grown HMEC from 7 different individuals ranging in age from 16-66 in our new media formulations, M85+X or M87A+X. In conjunction with Mark LaBarge in Mina Bissell's lab at LBNL, these cultures have been examined for lineage

markers by immunohistochemistry, immunofluorescence, and/or FACS sorting, using lineage specific antibodies (Figs. 2-3). The results indicate that cells with markers of myoepithelial (e.g., CD10, keratin 14), luminal (e.g., muc1, keratin 18 and 19, EpCam, Prom1/CD133) and progenitor lineages are present in these populations, with some variability depending on the specimen donor and passage level. To our knowledge, this represents the most active, long-term growth of luminal HMEC under simple culture conditions ever achieved. We have recently FACS sorted the heterogeneous populations into ongoing cultures of purified myoepithelial and luminal populations. In future years, we plan on using these purified populations as starting material for oncogene-induced transformation. In conjunction with Joe Gray's ICBP program at LBNL, the pre-stasis HMEC from 4 individuals have been analyzed for gene expression at different passage levels using the LBNL HTA μarray core facility. These results are consistent with the immunologic data, and also indicate that the milk-derived cells represent a near pure luminal population. In the future, we will additionally examine gene expression using the lineage-purified populations.

The effect of c-myc overexpression on different types of HMEC. Our previous studies had noted that overexpressed c-myc had little detectable effect on telomerase expression or immortalization of post-selection p16(-) HMEC (i.e., cells that had silenced p16 after growth in a high stress serum-free medium) from specimen 184. We have focused on the effects on telomerase and immortalization since our previous work has suggested that this is a key rate-limiting step in carcinogenesis (Stampfer and Yaswen, 2003, Stampfer et al., 2003). Our collaborator, Mark Jackson from Case Western, has since shown a similar result for post-selection HMEC from specimen 48 (Figure 4). Yet, our previous very preliminary study had indicated that pre-stasis HMEC grown in a lower stress medium were more vulnerable to c-myc immortalization and telomerase upregulation. Indeed, early passage pre-stasis 184 HMEC that had overcome stasis due to transduction of shRNA to p16 (and had thereby never experienced much culture stress) were apparently uniformly immortalized by c-myc. Thus, the known absence of p16 expression in post-selection HMEC, by itself, could not account for the inability of these cells to be myc-immortalized. A key goal of this proposal is to understand the mechanism underlying this distinction, since upregulation of telomerase is so critical a step in breast carcinogenesis. Additional, we want to know if different cell types cells immortalized by different means (e.g., pre-stasis or post-selection, with different experiences of stress, with different oncogenes, with different lineage markers) will give rise to immortalized lines of varying phenotype – not just basal as has been seen thus far.

In the past year we have examined the effect of c-myc overexpression on different HMEC types. In particular, we looked at our post-stasis Extended Life (EL) cultures derived following chemical carcinogen exposure of primary 184 HMEC cultures grown in the moderate stress medium, MM (Stampfer and Bartley, 1985, 1988). We chose three independently derived post-stasis EL populations, 184Aa, 184Be, and 184 Ce, which were already known to lack p16 expression (mutation in 184Aa, promoter methylation in 184Be and 184Ce). Previous work had shown that the 184Aa and 184Be, but not 184Ce populations, can give rise to rare clonal immortal lines following the genomic instability generated by the telomere dysfunction senescence barrier (Stampfer and Bartley 1985, Romanov et al., 2001, Stampfer et al. 2003, and unpublished). We hoped these results would elucidate which variables were associated with the ability of c-myc to immortalize (see Table 1). As shown in Figure 5, all three EL cultures were readily, and apparently uniformly, immortalized by c-myc. These results indicate that not just loss of p16 expression, but also p16 promoter methylation per se is not the responsible variable in determining whether post-stasis HMEC are vulnerable to myc-induced immortalization.

We are beginning to set up a more controlled experiment on the effect of media stress as a responsible variable. To this end, early and late passage cultures of 184 and 48R HMEC will be transduced first with p16sh, and then c-myc, to determine if cells that are closer to stasis, and have experienced more stress, will be more refractory to transformation. We know that the early passage pre-stasis HMEC can express some telomerase activity, which is reduced at later passages (Figure 6). Thus far, we have generated large stocks of passage 3 pre-stasis HMEC from specimens 184 and 48R in the low stress M87A+X medium. Populations from each of these specimens were sequentially transduced at 3p with eGFP and then either sh-eGFP or sh-p16. The transduced cultures have been selected with G418 (eGFP) and puromycin (sh-eGFP and sh-p16) and stocks of these cells at 5p have been

frozen in liquid nitrogen. In preliminary evaluations of the transduced HMEC, the sh-p16 transduced cells proliferated faster than the sh-eGFP transduced cells indicating effective knockdown of p16 expression. This needs to be confirmed by long term growth of the cultures as well as by immunostaining and quantitative RT-PCR.

Epigenetic changes associated with different HMEC types and pathways to transformation. We have initiated a collaboration with Bernie Futscher at the UACC to study the epigenetic modifications associated with normal and transformed HMEC, and to see if these modifications may vary in association with other parameters, such as ability of c-myc to induce telomerase activity and immortalization, and different pathways to various transformed phenotypes. Our preliminary studies have examined global promoter methylation changes using a μarray with 13,000 human promoters and a methylcytosine antibody. Little or no differences were seen among pre-stasis HMEC from different individuals, at different passages, or grown in different media (M85+X vs serum-free MCDB170). A large number of differences were seen between the pre-stasis HMEC and their isogenic p16(-) post-selection cultures. This result, as well as similar data for gene expression changes (Li et al., 2007, Garbe et al., in prep), point out the non-normal nature of these post-selection HMEC, which are currently being sold commercially and used as "normal" HMEC. Interestingly, post-stasis HMEC that had overcome the stasis barrier by other means, such as the EL cultures above, showed significantly fewer differences from the pre-stasis HMEC, and the population that had p16 expression reduced through the p16sh had few differences from the pre-stasis. Thus it appears that (1) epigenetic changes occur very early in the process of transformation, in still finite populations, and (2) different pathways to overcoming the stasis tumor-suppressive senescence barrier are associated with differing epigenetic changes. Further changes were associated with the c-myc immortalized derivatives of the EL cultures.

The effects of different oncogenes on the pathways to transformation of unstressed pre-stasis HMEC. Our lab has deferred starting these experiments in the first year, while setting up further collaboration with Mark Jackson of Case Western on these studies. We hosted Mark at our lab for a week in March, to train him on the techniques involved in growing primary HMEC cultures from organoids, and growth of the pre-stasis HMEC in low stress media. He has begun experiments to try to alter ERα activity in the organoids cultures as a possible way to generate ER(+) transformed cells (i.e., of luminal subtype) using endogenous ERα rather than expression of an exogenous transgene.

KEY RESEARCH ACCOMPLISHMENTS:

• Developed method for long-term growth of normal pre-stasis HMEC which express markers of multiple HMEC lineages, as in vivo. Demonstrated that these heterogeneous populations can be FACS sorted into purified luminal and myoepithelial lineage populations

• Shown that the ability of c-myc overexpression to immortalize finite HMEC depends upon (still undefined) parameters of the starting population, but not on expression or silencing of p16.

• Shown that global promoter methylation patterns vary significantly along different pathways to transformation, but not based on expression or silencing of p16.

REPORTABLE OUTCOMES:

Garbe, JC, Jackson, MW, Stampfer, MR, Vulnerability of Normal Human Mammary Epithelial Cells to Oncogenic Transformation. Department of Defense Breast Cancer Research Program Era of Hope Meeting, June 2008, Baltimore, MD.

CONCLUSIONS:

We believe that pathologically relevant HMEC models are of great value for both basic research into understanding cancer etiology and progression, and for developing and testing novel methods for clinical intervention. This proposal seeks to enhance the usefulness of our existing HMEC culture system by creating a more complete experimentally tractable model of the diversity of breast cancer etiologies and pathways to malignancy, as well as by generating new cellular reagents for dissemination. Towards this goal, in the past year we have demonstrated that normal HMEC reflective of the various in vivo lineages (e.g., luminal and myoepithelial) can be cultured for up to 60 PD, and FACS isolated based on lineage markers. These cells will be used for future experiments involving oncogene-induced transformation, to determine what starting phenotype may be most likely to yield transformed cells that have other than a basal phenotype (the phenotype seen thus far resulting from in vitro transformation). We have also seen that the ability of the c-myc oncogene to induce immortalization varies depending upon the phenotype of the starting HMEC population, and are trying to determine what constitutes the relevant variable; we now know that it does not involve p16 expression or silencing, and are preparing for experiments that will examine the role of stress exposure. We have also begun studies to determine if differing pathways to transformation and vulnerability to myc-immortalization can be correlated with specific epigenetic changes; our preliminary results suggest that early epigenetic changes may be associated with specific phenotypes.

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APPENDICES:
Table 1
Figures 1-6

Table 1: What variables determine whether a post-stasis HMEC will or will not respond to transduction of c-myc by induction of telomerase activity and immortalization (myc is a transactivator of hTERT).

All the starting cells in this table are post-stasis, but they became post-stasis by different means and having been exposed to different variables. Variables we are considering:
1) The experience of stasis-type stress (that elevates p16 expression) can influence the chromatin landscape or other parameters that prevent myc transactivation of TERT. That would require either that all 3 EL cultures below had seen low stress – possible but unlikely, or that changes only occur after ongoing high stress.
2) The stress-inducing serum-free MCDB170 medium induces the HMEC towards a different lineage, or different kinds of stressful experience at stasis, than is seen in cells grown in the serum-containing media. Their morphology at stasis is quite different, looking like more mature myoepithelial (with stress fibers).
3) The mechanism by which p16 is silened in the high stress serum-free medium during selection has collateral (perhaps epigenetic) effects that differ from the mechanism by which p16 is silenced following benzo(a)pyrene exposure, and results in a phenotype more refractory to myc-immortalization.

Cell Type	**myc immortalize?**	**p16 status/how**	**experience of stasis**	**pre-stasis medium**
184B; 48RS post-selection	- or **very** rare	silenced stressful medium	total	MCDB170 serum free
184Aa Extended Life	~uniform	mutated B(a)P	likely significant but can't be sure	MM, low serum, moderate stress
184Fp16sh post-stasis	~uniform	inactivated with shRNA	low	M85 lower stress
184Be Extended Life	~uniform	silenced B(a)P	likely significant but can't be sure	MM, low serum, moderate stress
184Ce Extended Life	~uniform	silenced B(a)P	likely significant but can't be sure	MM, low serum, moderate stress

Figure 1. Growth of pre-stasis HMEC in low stress media. Primary cultures from three different reduction mammoplasty specimens (184, 48R, 240L) were started from organoids and grown in M85+X, or M87A+X. The number of PD in primary culture cannot be accurately determined (likely ~5-15 PD); growth is shown starting from passage 2. Note the rapid sustained growth until near stasis. 250MK are cells derived from milk that were grown in MM for primary culture, and switched to M85+X at second passage. These data indicate that it is possible to generate large standardized stocks of pre-stasis HMEC from individual specimen donors at passages 3-5.

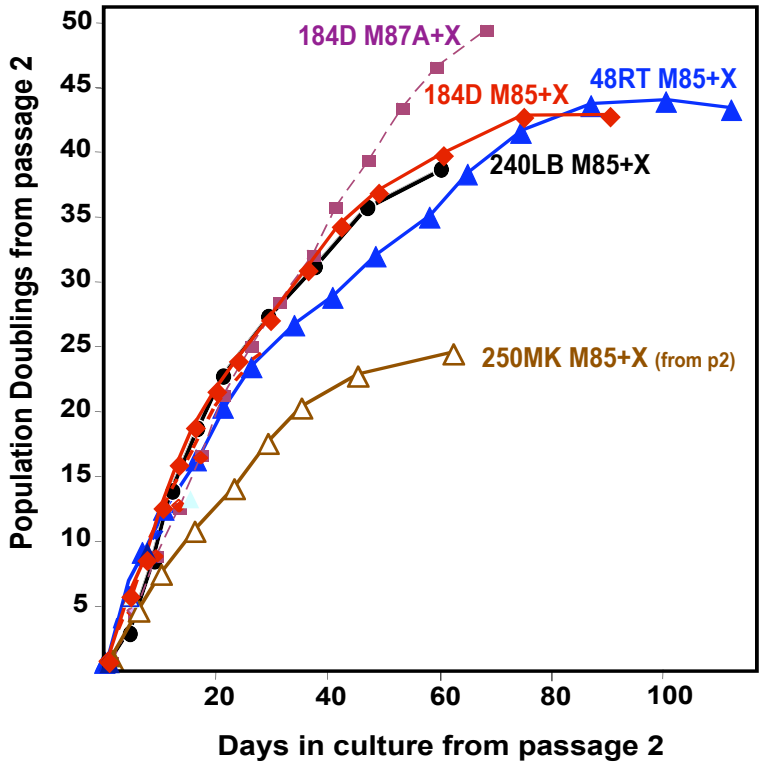

Figure 2. Expression of lineage markers in pre-stasis HMEC. A. Lineage markers in 48RT passage 5 stained by IF showing cells with myoepithelial lineage marker (K14) with some interspersed cells displaying luminal markers (K19, EpCam) and small patches homogeneous for luminal markers. B. Lineage markers in milk-derived 250MK at passage 3 indicate that most cells have a luminal phenotype.

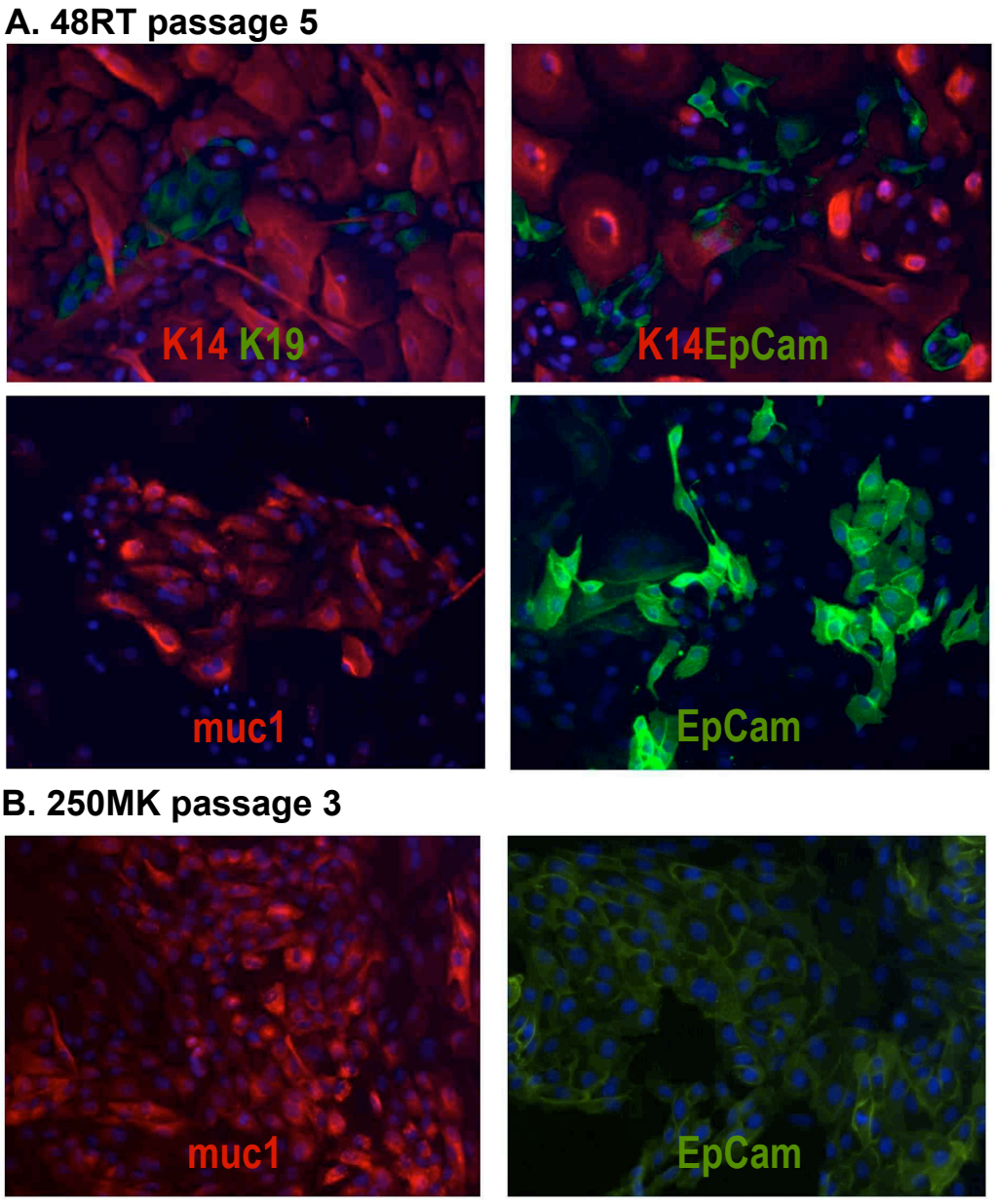

Figure 3. FACS sorting of pre-stasis HMEC by lineage markers. HMEC from specimen 240L were grown in M87A+X medium and flow sorted at passage 5 using fluorescently labeled antibodies to the luminal markers EpCAM and CD133 (PROM1), and the basal marker CD10. Sorted populations were replated in culture and representative images obtained. Sorted cells show distinct morphological differences, with the luminal population (CD10-CD133+EpCam+) resembling luminal cultures obtained from milk cells and the myoepithelial population (CD10+CD133-EpCam-) resembling prior cultures of mostly myoepithelial lineages.

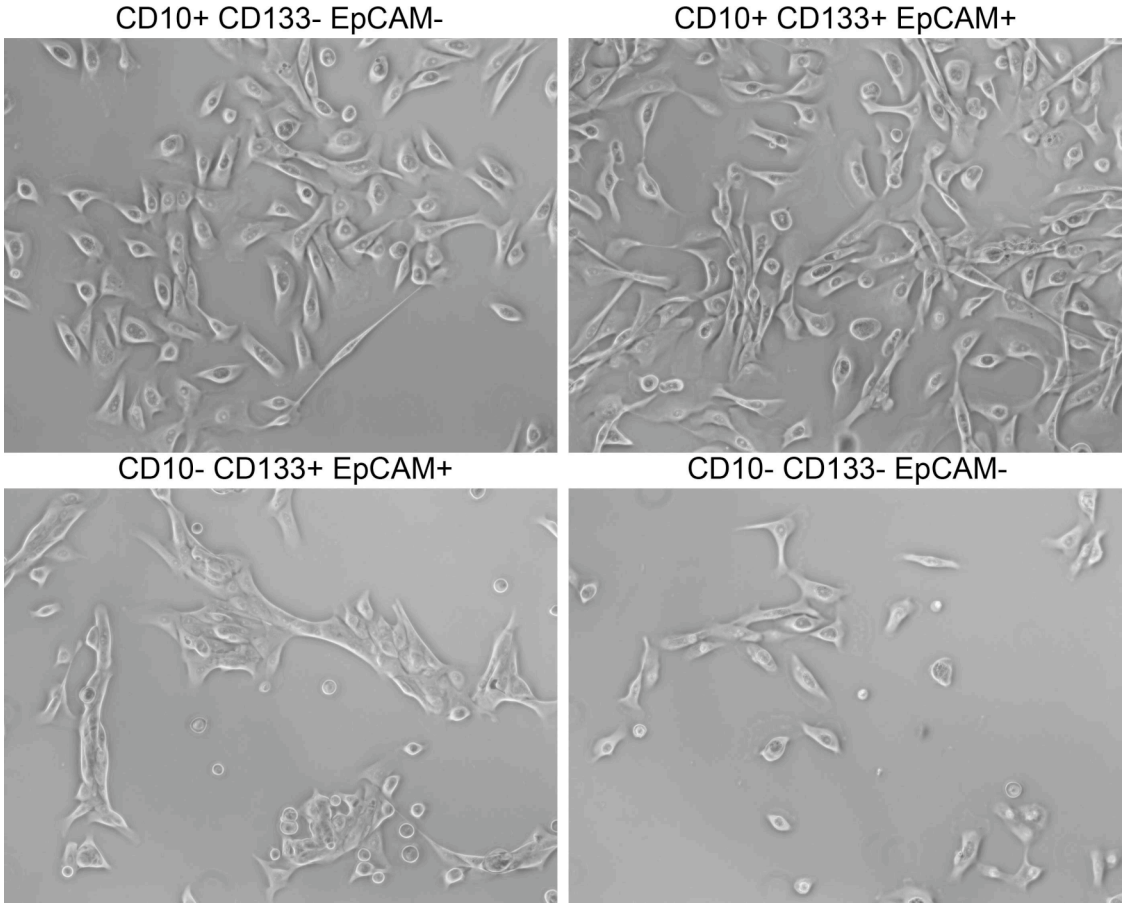

Figure 4. Transduction of c-myc into post-stasis post-selection HMEC very rarely produces immortally transformed cells. Finite lifespan, post-selection 184 and 48R HMEC grown in serum-free MCDB170 medium were transduced with c-myc containing retrovirus or empty vector control. In 10 independent experiments transducing c-myc into post-selection 184 HMEC, only one clonally derived immortalized line was obtained. TRAP assays showed little or no telomerase activity following c-myc transduction. Control untreated post-selection 184B show fewer PD prior to telomere dysfunction senescence than 48RS.

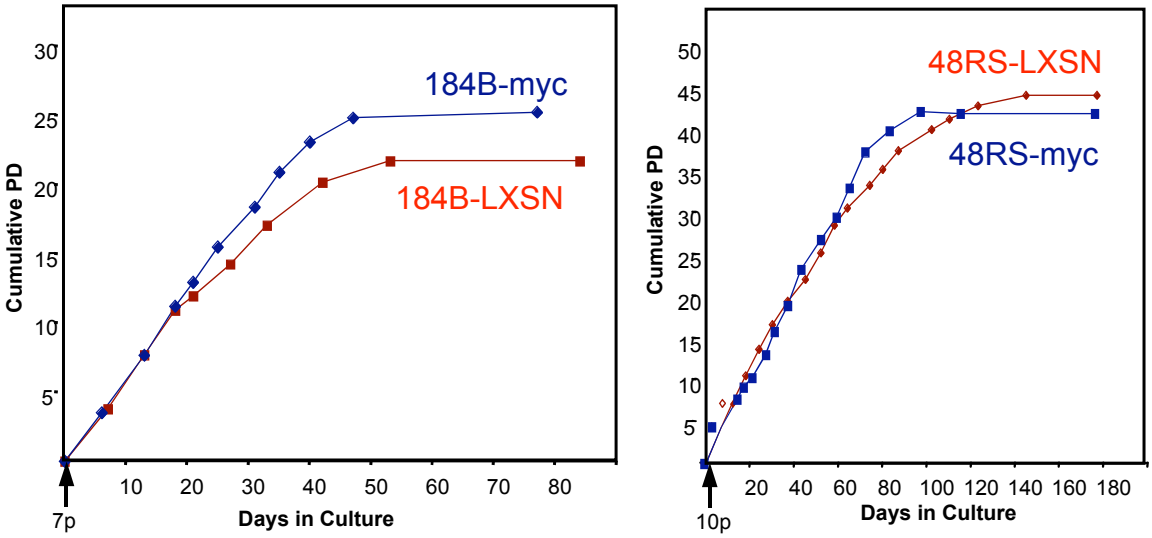

Figure 5. Transduction of c-myc into post-stasis EL HMEC efficiently produces immortally transformed cells. Extended life 184Aa (p16 mutated), 184Be and 184Ce (p16 promoter methylation) HMEC were transduced with c-myc containing retrovirus or with empty vector control at the indicated passage levels. All myc-transduced populations showed rapid increases in growth rate and no evidence of clonal selection for immortalized cells. Rapid induction of telomerase activity as measured by TRAP assays has been shown for 184Aa-myc and immortalized cultures were obtained in 7/7 independent experiments. Control untreated EL 184Be show few PD prior to telomere dysfunction, while 184Aa and 184Ce show somewhat more proliferative potential.

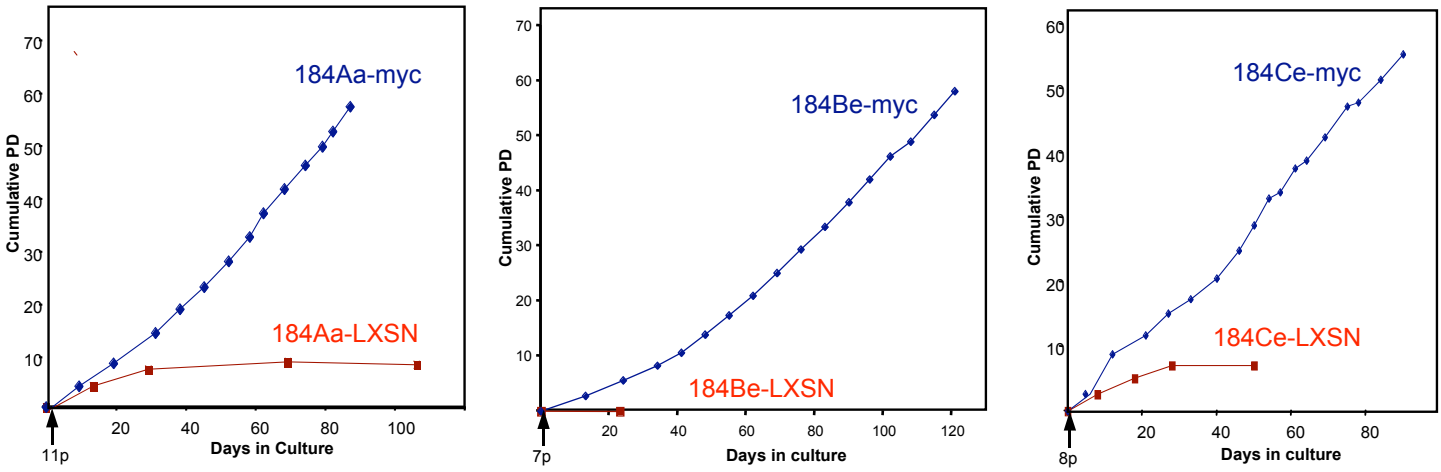

Figure 6. TRAP assay showing the presence of telomerase activity in pre-stasis HMEC. Cultures of pre-stasis HMEC from specimen 184 were grown in low stress medium M85±X, harvested, and prepared as extracts. Protein extracts were assayed for telomerase activity using the TrapEZE telomerase detection kit and reaction products separated by PAGE. The stained gel shows telomerase activity in the early passage cultures. In comparison the immortal HMEC line 184A1 and the OTP control extracts show strong telomerase activity while later passage pre-stasis and post-selection cells (184v) show weak, if any, activity.