

# Disparate effects of telomere attrition on gene expression during replicative senescence of human mammary epithelial cells cultured under different conditions

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**Telomere shortening in populations of human mammary epithelial cells (HMECs) that survive early replicative arrest (M0) by the inactivation of p16<sup>INK4A</sup> during cell culture on plastic dishes leads to a state of permanent replicative arrest termed senescence. While culture of HMECs on feeder layers abrogates M0 and p16<sup>INK4A</sup> inactivation, progressive telomere attrition in these cells also eventually results in permanent replicative arrest. Expression of telomerase prevents both senescence on plastic (S-P) and senescence on feeder layers (S-FL) in HMECs, as it does also in cultured primary human fibroblasts. We report here that the gene expression profiles of senescence in HMECs of the same lineage maintained under different culture conditions showed surprisingly little commonality. Moreover, neither of these senescence-associated profiles in HMECs resembles the profile for senescence in human fibroblasts. These results indicate that senescence-associated alterations in gene expression resulting from telomere attrition are affected by culture conditions as well as by cell origins, and argue that replicative senescence at the molecular level is a diverse rather than unique cellular process.**

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## Introduction

The inability of normal somatic cells in culture to proliferate indefinitely as reported initially by Hayflick and Moorhead (1961) is one of the many differences between normal cells and cancer cells. This property,

termed replicative senescence, has been proposed to provide a barrier against the accumulation of multiple mutations required for the development of cancer (Sager, 1991; Wright and Shay, 2001). As most normal somatic cells lack the activity of telomerase (Kim *et al.*, 1994), the enzyme responsible for maintaining telomere length (Greider and Blackburn, 1987), progressive telomere shortening occurs during each cell division, truncating telomeres to a critical length or creating an altered telomere state that signals events leading to senescence (Harley, 1991; Holt *et al.*, 1996; Blackburn, 2001; Karlseder *et al.*, 2002; Masutomi *et al.*, 2003; Stewart *et al.*, 2003). The expression of the telomerase catalytic subunit (hTERT) in primary cultures of human fibroblasts and other human somatic cells has been shown to abrogate senescence-related growth arrest (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998; Yang *et al.*, 1999; Dickson *et al.*, 2000; Hooijberg *et al.*, 2000; Rufer *et al.*, 2001). The ability of telomere length to modulate replicative senescence distinguishes this state mechanistically from quiescence and other telomere-independent mechanisms that lead to the arrest of cell proliferation (Bodnar *et al.*, 1998; Wright and Shay, 2001).

In contrast to primary cultures of human fibroblasts, populations of human mammary epithelial cells (HMECs) propagated under normal conditions (i.e. on plastic dishes containing chemically defined medium) for several population doublings show increased expression of the cyclin-dependent kinase inhibitor p16<sup>INK4A</sup> (p16) and undergo a type of telomere-independent growth arrest termed M0 (Romanov *et al.*, 2001; Yaswen and Stampfer, 2001). A subset of cells within this population experience inactivation of p16 and continue to proliferate, eventually entering a permanent growth arrest state, termed M1, that is dependent on telomere attrition (Kiyono *et al.*, 1998; Romanov *et al.*, 2001). This senescence state can be distinguished from a telomere-dependent state of population mortality (M2 or crisis) observed in HMECs that adventitiously express the E6 and E7 oncoproteins of human papilloma virus (Kiyono

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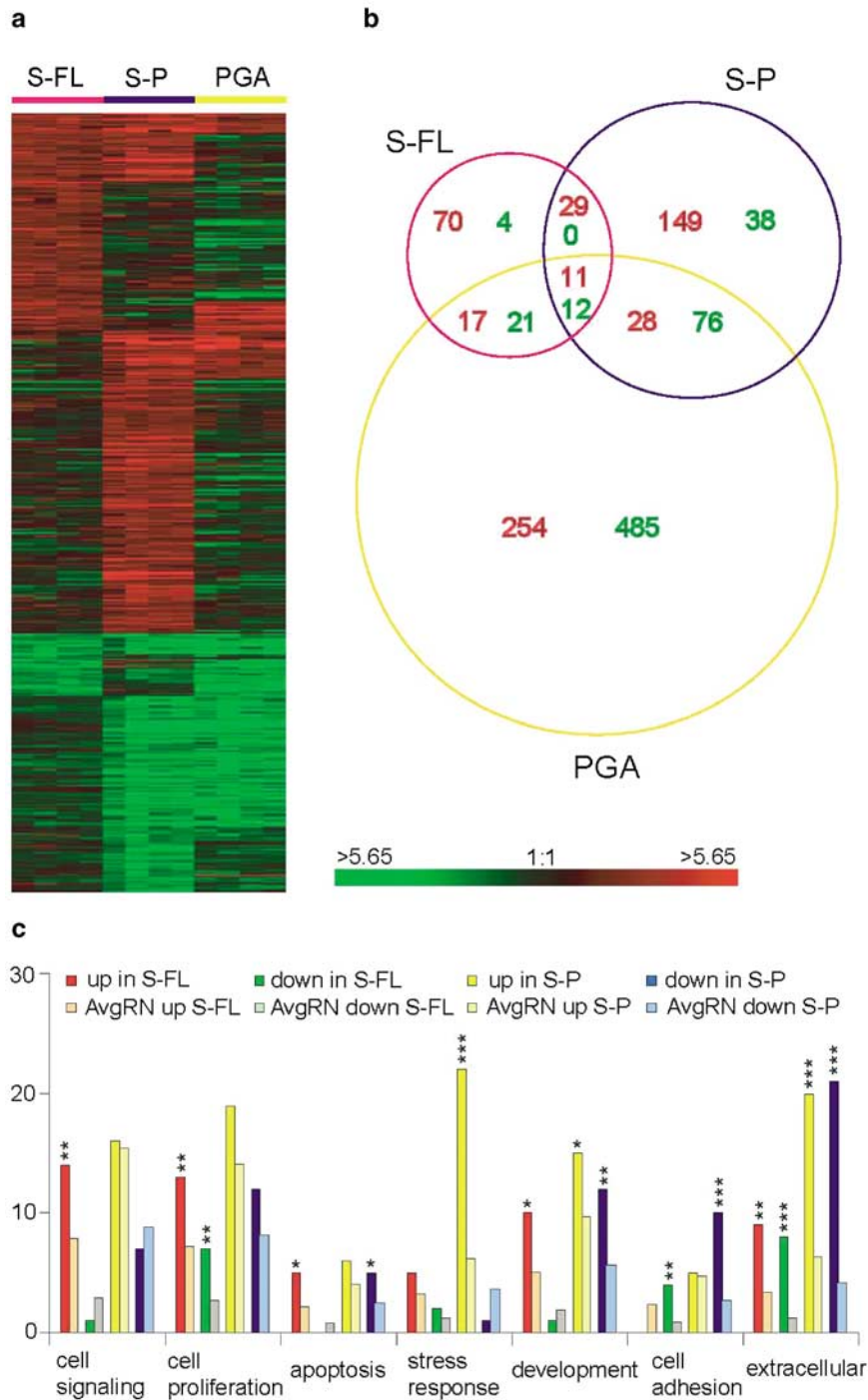
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*et al.*, 1998). Recent evidence indicates that populations of HMECs cultured on fibroblast feeder layers do not exhibit p16-related premature growth arrest (PGA) (i.e. M0), suggesting that M0 is a consequence of suboptimal culture conditions (Ramirez *et al.*, 2001). However, like HMECs cultured on plastic, populations of HMECs cultured on feeder layers show continued telomere shortening and eventually enter a state of irreversible proliferative arrest; like senescence in HMECs cultured on plastic (Kiyono *et al.*, 1998), this state of replicative

arrest of HMECs on feeder layers can be abrogated by the expression of hTERT (Ramirez *et al.*, 2001; Herbert *et al.*, 2002).

Recent studies using cDNA microarrays to assess changes in gene expression associated with telomere shortening during replicative senescence in human fibroblasts or during M1 in HMECs cultured on plastic indicate that these two senescence events generate different gene expression signatures (Zhang *et al.*, 2003). Here, we report investigations that globally



compare gene expression in HMECs during the telomere-dependent states of senescence occurring on feeder layers (S-FL) and on plastic (S-P). We found that gene expression profiles of senescence in HMECs of the same lineage cultured under different conditions showed surprisingly little commonality. Moreover, neither profile resembles the replicative senescence signature of fibroblasts. Our results indicate that alterations in gene expression occurring during replicative arrest secondary to telomere attrition depend significantly on the cell culture conditions employed for cell growth during and after the period of telomere shortening.

## Results

### *Distinctive gene expression profiles of HMECs at senescence grown on plastic or feeder layers*

We cultured a primary population of HMECs that were derived from organoid culture on feeder layers (mitomycin C-treated NIH3T3 cells) and continuously passaged them. These cells encountered only one growth barrier that arrests proliferation due to replicative senescence. In contrast, cells from the same lineage cultured under ordinary conditions (on plastic dishes containing chemically defined medium) typically encountered an initial growth barrier (i.e. M0), emerged from this barrier through a biological 'selection' process including inactivation of p16 and after approximately 30–35 population doublings, reached replicative senescence (i.e. M1) (Herbert *et al.*, 2002). For clarity, we use S-FL and S-P to distinguish these two states of permanent replicative arrest in HMECs occurring on feeder layers and on plastic, respectively. Both the S-FL and S-P states can be prevented by the expression of hTERT (Kiyono *et al.*, 1998; Ramirez *et al.*, 2001; Herbert *et al.*, 2002), indicating that progressive telomere shortening is the primary cause. The mean telomere length of the HMECs at S-P in this study was 4.1 kb, which was determined to be similar to the length (5.1 kb) at S-FL (Ramirez *et al.*, 2003).

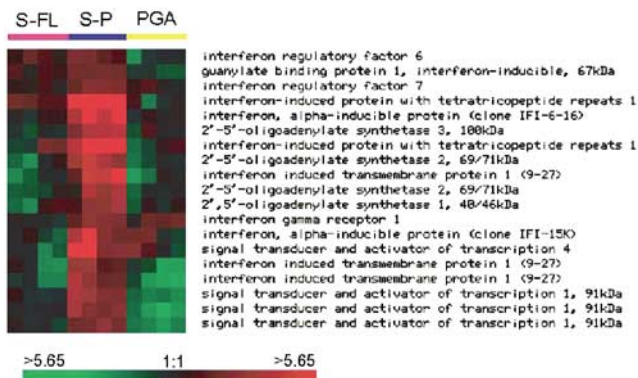
High-density cDNA microarrays were used to compare global gene expression patterns in both states of replicative arrest in HMECs. Poly(A) RNA was

extracted from senescent HMECs in both states, and cDNA corresponding to these RNAs was fluorescently labeled and hybridized together with differentially labeled cDNA from poly(A) RNA of early passage HMECs of the same lineage cultured on feeder layers. Hybridization results were assessed quantitatively at a single gene resolution and analysed as described previously (Zhang *et al.*, 2003). Notwithstanding evidence that both S-FL and S-P in HMECs are controlled by progressive telomere shortening (Kiyono *et al.*, 1998; Ramirez *et al.*, 2001; Romanov *et al.*, 2001; Herbert *et al.*, 2002), their gene expression profiles were distinctly different (Figure 1, detailed annotations for genes showing altered expression is provided in Table 1 of Supplementary Information). Only a limited percentage of transcriptional alterations was observed in common to these two senescence states (Figure 1b).

Genes whose expression was altered during either senescence state in HMECs were further classified according to their annotated role in biological processes or their cellular locations, as retrieved from Gene Ontology (<http://www.geneontology.org>). Enrichment of a particular class of genes was determined by comparison with randomly selected gene lists of the same size. As shown in Figure 1c, Unigenes-encoding proteins having certain annotated functions were represented differentially and disproportionately in these two states of replicative arrest in HMECs. For example, extracellular protein-encoding genes were significantly enriched among genes showing altered expression during both S-FL and S-P states. Genes involved in cell proliferation were significantly enriched among those altered during S-FL but not during S-P. Conversely, genes related to the stress response were significantly enriched among the loci upregulated during S-P but not during S-FL.

Interferon response genes were among the stress response loci prominently induced during S-P (Figure 2). To learn whether culture on plastic is sufficient to induce the expression of interferon response genes, we transferred proliferating early passage HMECs being cultured on feeder layers to plastic dishes containing chemically defined medium. As shown in Figure 2, the population of cells that was shifted to plastic dishes lacking a feeder layer showed no increase

**Figure 1** Genes showing altered expression during senescence in HMECs. (a) Comparison of altered gene expression of HMECs on feeder layers at senescence (S-FL), HMECs at senescence grown on plastic culture dishes (S-P) that had escaped M0 and thus have inactivated p16, and premature growth arrest (PGA) which similar to M0 is the cessation of proliferation of early passage HMECs after being transferred onto plastic dishes from feeder layers. Poly(A) RNA from senescent cells or premature arrested cells was labeled with Cy5-dUTP (red), mixed with Cy3-dUTP (green)-labeled poly(A) RNA from early passage proliferating cells cultured on feeder layers and hybridized to cDNA microarrays. Data values for experiments in which labelings were reversed (dye-swap) were inverted before analysis. Rows represent genes (IMAGE clones) and columns represent separate repetitions using the same RNA sample in which individual samples were tested. IMAGE clones that showed alteration of gene expression are displayed using a pseudocolor visualization matrix (Eisen *et al.*, 1998). The expression ratio is shown by color and intensity according to the color scale. The portion of the dendrogram that represents gene expression alterations unique to PGA is not shown. Complete lists of detailed gene annotations are provided in Table 1 of Supplementary Information. (b) Summary of the numbers of genes whose expression was altered in HMECs during S-FL, S-P and PGA. Numbers in red represent upregulation and those in green represent downregulation. (c) Genes showing altered transcription during both S-FL and S-P in HMECs were classified according to their gene ontology annotations of biological processes and cellular locations. Only the categories that showed significant enrichment as compared to the average number of randomly selected genes (AvgRN) were shown. The likelihood of enrichment of genes in a group due to random events (*P*-value) is indicated by asterisks: \**P*<0.05; \*\**P*<0.01 and \*\*\**P*<0.001



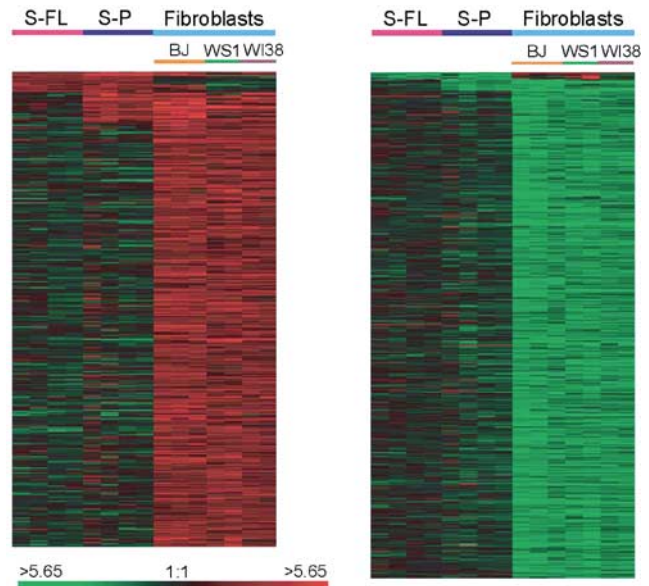
**Figure 2** Induction of interferon response genes during S-P in HMECs. See Figure 1 for experimental details

in the expression of interferon response genes, indicating that culture on plastic *per se* is not sufficient for the induction of these genes. These data further argue that the induction of interferon response genes is a physiological concomitant of S-P state in HMECs. Consequent to the shift from feeder layers to plastic, HMECs stopped dividing after only six to 10 population doublings and entered a state of proliferative arrest that resembles M0 growth arrest, and which we termed PGA. The global gene expression profiles of these HMECs were distinct from the profiles for S-FL and S-P (Figure 1, detailed annotations for genes showing altered expression is provided in Table 1 of Supplementary Information).

#### *Disparate gene expression profiles of senescence in HMECs and fibroblasts*

Unlike HMECs in S-P, HMECs cultured on feeder layers enter a telomere-dependent state of replicative senescence with their p16 gene expression intact, as it occurs for fibroblasts entering replicative senescence (Ramirez *et al.*, 2001; Herbert *et al.*, 2002). We therefore wished to determine whether the gene expression profile of S-FL in HMECs is less divergent from senescent fibroblast profile than is the profile of S-P (Zhang *et al.*, 2003). In these experiments, we determined gene expression alterations of both S-FL and S-P relative to early passage HMECs cultured on feeder layers, and compared these with the changes occurring in senescent fibroblasts *vs* early passage fibroblasts.

The expression data for fibroblasts were retrieved from our previous study (Zhang *et al.*, 2003). However, as the microarrays used in the previous work had 10 000 fewer cDNA spots than were present on our current microarray slides, we analysed only spots common to the two sets of microarrays. Significantly, we found that alterations in gene expression occurring during senescence in p16-proficient HMECs also had little in common with those of similarly p16-proficient senescent fibroblasts (Figure 3, detailed annotations are provided in Table 2 of Supplementary Information): only five genes (DOC1, RAI3, SERPINB2, PLAU, AKAP12) –



**Figure 3** Distinctive gene expression alterations during senescence in HMECs and fibroblasts. Data for senescence in fibroblasts were retrieved from previously published study using three different lines (BJ, WS1 and WI38) (Zhang *et al.*, 2003). Detailed gene annotations are provided in Table 2 of Supplementary Information. See Figure 1 for experimental details

three of which (DOC1, RAI3, SERPINB2) were also altered in S-P – showed similar alterations.

Previously, we found that genes positively regulating cell cycle progression are significantly enriched among transcripts altered during senescence in human fibroblasts (Zhang *et al.*, 2003). However, such alterations were absent from HMECs in either S-FL or S-P (Figure 3). Consistent with a previously reported finding (Romanov *et al.*, 2001), we found that senescent HMECs cultured either on feeder layers or on plastic were arrested almost equally in both G1/G0 and G2/M (data not shown), contrasting with the G1/G0 cell cycle distribution of senescent human fibroblasts.

## Discussion

Our results indicate that gene expression alterations occurring during permanent replicative arrest associated with telomere attrition in HMECs are strongly affected by the conditions used to culture these cells. Our finding that relatively few alterations occurred in common in HMECs entering the senescence state on feeder layers or plastic, and between senescence in HMECs and senescence in fibroblasts, argues that telomere-associated replicative senescence is a diverse, rather than unique process. These findings are consistent with earlier evidence of multiple senescence-related complementation groups and with the conclusion that senescence can occur by multiple pathways – any one of which is sufficient to confer the senescence phenotype (Pereira-Smith and Smith, 1988).

The term senescence has been used to describe proliferative arrest of cell cultures in response to overexpression of oncogenes (Serrano *et al.*, 1997; Lin *et al.*, 1998; Zhu *et al.*, 1998; Dimri *et al.*, 2000), exposure to oxidative or DNA damage (Di Leonardo *et al.*, 1994; Chen *et al.*, 1995; von Zglinicki *et al.*, 1995; Robles and Adami, 1998), histone deacetylase inhibitors (Ogryzko *et al.*, 1996) or suboptimal cell culture conditions (Sherr and DePinho, 2000; Ramirez *et al.*, 2001; Herbert *et al.*, 2002). An impediment to investigations of replicative senescence at the genetic or molecular level has been a lack of reliable cellular or molecular markers that distinguish replicative senescence from other processes that affect cell cycle progression, as growth arrest *per se* is not an accurate measurement of replicative senescence (Sherr and DePinho, 2000; Wright and Shay, 2001, 2002). Our discovery that populations of cells of the same lineage cultured under different conditions as well as populations of different cellular origins show only limited commonality of gene expression profiles when entering replicative senescence following telomere attrition argues that the process by which cells undergo irreversible proliferative arrest (i.e. replicative senescence) is heterogeneous – rather than unique. These findings further highlight the value of global measurements of gene expression (Zhang *et al.*, 2003), rather than phenotypic end points, in studying this complicated process.

## Materials and methods

### Cell cultures

HMECs, derived from a 33-year-old patient undergoing augmentation mammoplasty, were cultured either on mitomycin C-treated NIH3T3 feeder layers or under standard conditions as described (Ramirez *et al.*, 2001; Herbert *et al.*, 2002). Briefly, the cells were grown in MCDB 170 media (Invitrogen) supplemented with 0.4% bovine pituitary extract

(Hammond Cell Tech, Alameda, CA, USA), 10 ng/ml epidermal growth factor (Invitrogen) and 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 5 µg/ml transferrin and 50 µg/ml gentamicin (all from Sigma Biochemicals). For the growth on feeder layers, HMEC media were supplemented with 1% cosmic calf serum (Hyclone, Logan, UT, USA). The medium was changed every 2–3 days. HMECs cultured on feeder layers were separated from feeder layers before RNA extraction.

### Microarray analysis

cDNA microarrays containing 41 805 IMAGE clones representing about 29 043 unique genes were obtained from Stanford Functional Genomics facility. Poly(A) RNA was extracted using a FastTrack 2.0 kit (Invitrogen) and labeled by reverse transcription using Superscript II enzyme (Invitrogen) and oligo(dT)<sub>18</sub> primer (New England Biolabs) in the presence of Cy3-dUTP or Cy5-dUTP (Amersham). Detailed RNA labeling, microarray hybridization, data collection, data normalization and data selection followed previously described procedures (Zhang *et al.*, 2003). Raw data were deposited into Stanford Microarray Database (<http://genome-www5.stanford.edu/MicroArray/SMD>). Data values for dye-swap experimental repeats were inverted. Prior to statistical analysis, missing values in the microarray data set were estimated with KNNimpute using 14 neighbors (Troynskaya *et al.*, 2001). Data were then analysed using modified *t*-score (Tusher *et al.*, 2001) to determine the significance of transcriptional alterations by the GABRIEL program (<http://Gabriel.stanford.edu>) (Pan *et al.*, 2002) as described previously (Zhang *et al.*, 2003).

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