Analysis of individual cells identifies cell-to-cell variability following induction of cellular senescence

Christopher D. Wiley, 1 James M. Flynn, 1 Christopher Morrissey, 2 Ronald Lebofsky, 2 Joe Šhuha, 2 Xiao Dong, 3 Marc A. Unger, 3 Jan Vig, 3 Simon Melov 1 and Judith Campisi 1,4
1 Buck Institute for Research on Aging, Novato, CA 94945, USA
2 Fluidigm Corporation, South San Francisco, CA 94080, USA
3 Department of Genetics, Albert Einstein College of Medicine, Bronx, NY 10461, USA
4 Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

Summary
Senescent cells play important roles in both physiological and pathological processes, including cancer and aging. In all cases, however, senescent cells comprise only a small fraction of tissues. Senescent phenotypes have been studied largely in relatively homogeneous populations of cultured cells. In vivo, senescent cells are generally identified by a small number of markers, but whether and how these markers vary among individual cells is unknown. We therefore utilized a combination of single-cell isolation and a nanofluidic PCR platform to determine the contributions of individual cells to the overall gene expression profile of senescent human fibroblast populations. Individual senescent cells were surprisingly heterogeneous in their gene expression signatures. This cell-to-cell variability resulted in a loss of correlation among the expression of several senescence-associated genes. Many genes encoding senescence-associated secretory phenotype (SASP) factors, a major contributor to the effects of senescent cells in vivo, showed marked variability with a subset of highly induced genes accounting for the increases observed at the population level. Inflammatory genes in clustered genomic loci showed a greater correlation with senescence compared to nonclustered loci, suggesting that these genes are coregulated by genomic location. Together, these data offer new insights into how genes are regulated in senescent cells and suggest that single markers are inadequate to identify senescent cells in vivo.

Key words: aging; cellular senescence; cytokines; single cell; transcriptomics.

Introduction
Cellular senescence is a process by which mitotically competent cells permanently arrest proliferation (growth) in response to a variety of physiological signals and pathological stresses (Munoz-Espin & Serrano, 2014). Because the growth arrest prevents the propagation of stressed or damaged cells, the senescence response is an important tumor-suppressive mechanism (Campisi, 2013). Further, because senescent cells accumulate with age, they can cause or contribute to several degenerative diseases of aging (Baker et al., 2016). These effects might stem from the fact that senescent cells cannot divide and therefore cannot create new cells to maintain tissue homeostasis. However, as senescent cells generally comprise a minority of cells within even very old tissues (Dimri et al., 1995; Herbig et al., 2006; Kreiling et al., 2011; Waaijer et al., 2012; Baker et al., 2016), it is more likely that senescent cells drive age-related disease cells nonautonomously. Indeed, senescent cells secrete a myriad of inflammatory cytokines, chemokines, proteases, and growth factors that can have potent effects on tissue microenvironments (Coppe et al., 2008) and thus drive age-related pathologies by mechanisms that extend beyond the loss of proliferative potential.

Traditional gene expression analyses that compare transcriptional profiles of cell populations are limited because they measure average of gene expression levels across the entire population. For example, two populations of 5000 cells each might show a twofold difference in the mRNA level of a particular gene, but this change could result from every cell expressing twice as much mRNA, or from a single cell expressing 5000 times more of that mRNA. The difference between these possibilities could have enormous phenotypic consequences in the context of a tissue. Single-cell approaches offer advantages over population studies because they can distinguish between these types of scenarios. Single-cell analyses also require fewer cells and therefore can be used to interrogate the phenotypes of rare cells, such as senescent cells produced during organismal aging.

Senescent cells display many qualities that make them desirable candidates for single-cell transcriptional analysis. As noted above, they are typically rare (Dimri et al., 1995; Herbig et al., 2006; Kreiling et al., 2011; Waaijer et al., 2012; Baker et al., 2016) and also often occur alongside nonsenescent cells. Further, many of the phenotypic differences between senescent cells and their nonsenescent counterparts are due to changes in mRNA levels, including mRNAs encoding many SASP factors (Coppe et al., 2008).

To assess the contributions of individual senescent cells to known senescent phenotypes, we conducted quantitative PCR analyses of single quiescent and senescent cells from cultured populations of human fibroblasts. From these analyses, we find that (i) virtually all senescent cells display a gene expression signature that distinguishes them from their quiescent counterparts; (ii) nonetheless, the expression of most genes is more variable in senescent cells compared to quiescent cells; and (iii) there are correlations among genes expressed by senescent cells, including those encoding SASP factors, that localize in genomic clusters. Together, the data demonstrate that senescent phenotypes are more variable than the transcriptional profiles of cell populations previously suggested.

Results
Single senescent cells can be identified by gene expression signatures
Although cellular senescence is marked by strong phenotypic and gene expression changes at the population level, less is known about how individual cells contribute to the overall gene expression pattern. We...
highly studied SASP factors, such as IL-1β, IL-6, IL-7, CXCL1-3, MMP1, percentage of senescent cells relative to quiescent cells (Fig. S2C). A few studies et al. in which the transcript was detected compared to transcript detection in senescent cells (Freund (Fig. S2C). For example, for comparison, fewer differences were seen in detection percentages were detected at higher percentage in the MI dataset (Fig. S2B). By these 71 transcripts were detected in at least 30% of the analyzed cells, expression (mRNA) levels in 235 quiescent and 229 senescent cells. (multiple melting curves), resulting in a dataset comprised of 71 gene associated with cellular senescence. Of the 96 genes analyzed, we these genes mainly based on prior knowledge of gene expression with many of them being detected in empty wells. In addition, we manually isolated (MI) 163 single senescent and 156 single quiescent cells.

We analyzed the cells for expression (mRNA levels) of 96 genes, including those associated with cell cycle arrest (12 genes), nuclear lamins (four genes), mitochondria and energy production (12 genes), the SASP (46 genes), signal transduction (four genes), and other senescence regulators (11 genes), in addition to seven control genes, the expression of which were expected to be relatively invariant (Data S1). We chose these genes mainly based on prior knowledge of gene expression associated with cellular senescence. Of the 96 genes analyzed, we excluded 25 as either nonamplifying (no or late Ct) or nonspecific (multiple melting curves), resulting in a dataset comprised of 71 gene expression (mRNA) levels in 235 quiescent and 229 senescent cells. These 71 transcripts were detected in at least 30% of the analyzed cells, with many of them being detected in > 60% of the analyzed cells (Fig. S2A). Although several transcripts (e.g., CXCL2, MMP8, IGFBP6) were detected at a higher percentage in the C1 dataset, most transcripts were detected at higher percentage in the MI dataset (Fig. S2B). By comparison, fewer differences were seen in detection percentages between senescent and quiescent datasets, with a few exceptions (Fig. S2C). For example, for LMNB1 gene expression—which declines in senescent cells (Freund et al., 2012)—there were fewer senescent cells in which the transcript was detected compared to transcript detection in quiescent cells. Conversely, TNFRS10C gene expression, which is induced in senescent cells (Coppe et al., 2008), was detected in a higher percentage of senescent cells relative to quiescent cells (Fig. S2C). A few highly studied SASP factors, such as IL-1β, IL-6, IL-7, CXCL1-3, MMP1, MMP10, and CCL13, were also detected at low percentages (Fig. S2A), limiting the types of analyses that could be performed on these factors.

Our initial analysis revealed few significant differences between the two cell populations primarily because senescent cells had a lower overall transcription abundance, possibly owing to less efficient lysis/amplification. After normalization (Livak et al., 2013), however, it was possible to identify transcriptional profiles that were consistent with transcriptome analyses of bulk cell populations (Fig. 1A). Normalization also resulted in more coordinated expression profiles between the C1 and MI pools of cells (Fig. 1B; Fig. S3A,B). Following normalization, MI cells showed 61 significantly altered genes during senescence, while C1 showed 31, presumably due to the smaller number of successfully amplified genes. Notably, 28 of the altered genes were shared between C1 and MI cells (Fig. 1C).

Using these datasets, we first asked whether senescent and quiescent cells were distinguishable at the single-cell level by gene expression signatures. Principal component analysis (PCA) allowed us to identify senescent cells by sorting the first and second principal components (consisting of a combination of expression values for 70 genes) (Fig. 2A, right panel). When plotted in two dimensions (X-Y axes), quiescent and senescent populations clustered independently (Fig. 2A, left panel). Senescent cells displayed a larger spread of values, consistent with more variability.

Using the expression of all 70 genes, linear discriminant analysis (LDA) correctly classified > 98% of cells as senescent or quiescent. Furthermore, expression of several genes individually strongly predicted senescence status. The two most predictive genes were TNFRSF10C and CDKN1A (Fig. 2B). TNFRSF10C encodes a secreted decoy receptor that prevents TRAIL-induced apoptosis (Sheridan et al., 1997). This gene is induced in response to genotoxic stress in a p53-dependent manner (Sheikh et al., 1999) and is a component of the SASP (identified by its alias TRAILR3) (Coppe et al., 2008). CDKN1A, which encodes p21Waf1, is a cyclin-dependent kinase inhibitor (CDKi) and primary mediator of both the transient and permanent (senescence) p53-induced G1 arrests (El-Deiry et al., 1993; Dulic et al., 1994). CDKN2A, which encodes p16Ink4a, is another CDKi, and common marker of senescence and aging (Beausejour et al., 2003; Krishnamurthy et al., 2004), but was not assayed in these analyses owing to primer failures. Reduced LMNB1 or HMGB1 expression also strongly predicted senescence. Both gene products are lost from the nuclei of senescent cells in a p53-dependent
Senescent cells display more variability in mRNA levels than quiescent cells

There is increased variability in selected mRNA levels among single cardiomyocytes from aged, compared to young, mice, and in mouse fibroblasts treated with H$_2$O$_2$ (Bahr et al., 2006). Both age and H$_2$O$_2$ increase the number of senescent cells. In agreement with these studies, many of the genes (51%) assayed here displayed significantly increased variability in mRNA levels among single cells following induction of senescence. This increased variability was not universal, however, with a few genes (7%) (such as CYR61 and SOCS3) showing lower variance in senescent compared to quiescent cells (Fig. 3A). Control genes, known to show little change in expression in senescent cells, generally displayed only minor changes in variance. For example, GAPDH displayed a nonsignificant ($P > 0.05$, Monte Carlo permutation test) decrease in variability, whereas ACTB variability increased slightly (Fig. 3A–C). Interestingly, CDKN1A also showed no significant increases in variance ($P > 0.05$), despite being induced in senescent cells (Fig. 3B).

Senescence-associated variability in gene expression could result from genomic rearrangements, as suggested for aged and H$_2$O$_2$-treated cells (Bahr et al., 2006), but also from changes in transcriptional activation or repression in senescent cells. LMNB1 mRNA levels, which decline in senescent cells (Freund et al., 2012), also showed increased variability (Fig. 3B) accompanied, as expected, by fewer transcripts with detectable amplification. Similarly, many SASP factors, such as IL-8 and IL-1A, showed strongly increased variability, with a subset of cells displaying a very high level of gene expression (Fig. 3B). This finding suggests the increase in mRNAs encoding SASP factors (Coppe et al., 2008) could result from contributions by only a subset of senescent cells at any time.

Gene expression correlations change during cellular senescence

Are cells that strongly express SASP factors such as IL-8 and IL-1A a subset of senescent cells, or do individual cells express these and other senescence-associated transcripts in variable quantities? To address these questions, we calculated correlation coefficients ($R^2$) for all genes, resulting from contributions by only a subset of senescent cells at any time.
transcript abundance and correlation among paired transcripts (Fig. 4B), suggesting that variability alone cannot account for these increases.

Of all genes that displayed highly altered correlations with senescence, CDKN1A (which was consistently induced in senescent cells; Fig. 3B) was most notable, displaying increased correlations with 25 gene transcripts (Fig. 4C). Furthermore, CDKN1A showed a substantial shift in its correlation patterns, losing correlation with some genes (Class 1) and gaining correlation with others (Class 2) (Fig. 4A).

As many SASP factors are strongly clustered in the genome (Coppe et al., 2010), we asked whether genomic location is linked to expression coregulation. Supporting this possibility, transcripts from the IL-1 cluster (IL-1A and IL-1B, promoters separated by ~50 kb), as well as the CXCL
Gene expression correlations in quiescent and senescent cells. (A) Heat maps of Pearson’s coefficients ($R^2$) between clusters of coexpressed genes in quiescent (left panel) and senescent (right panel) cells. Green indicates positive correlations, red indicates negative correlations, and empty cells indicate nonsignificant ($P > 0.01$) correlations. Two classes of directly (green) and indirectly (red) correlated expression patterns (labeled Class 1 and Class 2) appear across multiple genes. (B) Circular node network indicating changes in correlations between quiescent and senescent cells. Number of altered correlations increases from left to right. Green nodes indicate genes with increased variability in senescent cells, while red nodes indicate decreased variability; white nodes indicate no change. Connecting lines indicate whether correlations increase (green) or decrease (red) between genes. (C) Correlation ($R^2$) levels of multiple genes relative to CDKN1A. Violet bars indicate quiescent cells, whereas red bars indicate senescent cells. (D) Heat map of correlations ($R^2$) between genes in the CXCL (blue) and IL-1 (orange) clusters in quiescent (left) and senescent (right) cells, as in A.

Cluster (in order: IL-8, CXCL1, CXCL5, CXCL3, and CXCL2, separated in total by $\sim$360 kb), went from nonsignificant correlations to stronger, significant direct correlations, suggesting these genes were induced in a coordinated manner (Fig. 4D). By comparison, little to no correlation of expression was observed when the IL-1 cluster was analyzed against the CXCL cluster (Fig. 4D), which are on different chromosomes. These data suggest that genomic organization can influence gene expression changes in single cells.

Together, our correlation data indicate that, whereas the expression of many genes is coordinated under quiescent conditions, some senescence-specific gene expression processes appear to be regulated independently of each other.

Discussion

As senescent cells are relatively rare, even in tissues from aged animals (Dimri et al., 1995; Herbig et al., 2006; Kreiling et al., 2011; Waaijer et al., 2012; Baker et al., 2016), the ability to identify and study individual senescent cells is a unique opportunity to better understand...
the range of senescent phenotypes that contribute to their physiological and pathological effects (Munoz-Espin & Serrano, 2014). In this study, we highlight the ability to identify senescent cells by their gene expression profile, while also demonstrating that the gene expression profiles of senescent cells are highly heterogeneous. Despite this heterogeneity, genes that reside in closely linked loci appear to be coregulated during senescence.

Identifying senescent cells at the single-cell level is an important technological step for future studies, especially in human tissues. While transgenic mouse models now allow senescent cells to be identified and isolated from mouse tissues (Demaria et al., 2014), identifying senescent cells in human tissues remains difficult. Our study provides proof of principle that single-cell analyses have potential for identifying senescent cells from human tissues.

Our findings emphasize the risk of using a single biomarker to identify senescent cells, whether in culture or in vivo. We recommend using several makers—in our own studies, for example, we tend to use combinations of SA-Bgal activity, loss of LMNB1 expression, HMGB1 relocalization, p16^INK4a and/or p21^WAF1 expressions, and the expression of strongly upregulated SASP factors. As many inducers of senescence (e.g., telomere attrition, ionizing radiation, bleomycin, and oncogene activation) ultimately induce a DNA damage response, it is likely that many of the factors identified in this study are common to several senescence inducers. However, inducers of senescence that do not cause genotoxic stress (e.g., mitochondrial dysfunction-associated senescence) (Wiley et al., 2016) can have a distinct SASP and gene expression profile and so would need to be identified by those signatures.

Increased gene expression variability in senescent cells is consistent with studies showing such variability during aging and in response to oxidative stress (Bahar et al., 2008). Our findings show that senescence-associated mRNA levels can vary from cell to cell. For example, increases in CDKN1A mRNA were tightly clustered, possibly reflecting uniform p53 activation following genotoxic stress (bleomycin administration). By comparison, LMNB1 and many SASP factors, showing decreased or increased expression, respectively, displayed large variability in expression levels in senescent cells. These data suggest the mechanisms governing the expression of these genes are subject to more stochastic events than those that govern CDKN1A expression. Alternatively, genes that show large expression variability might fluctuate temporally, which, in an asynchronous population, would result in cell-to-cell differences in the expression levels at any given time.

The increased correlation between genes clustered within genomic loci suggests a level of gene regulation that has not previously been described for senescent cells. One possibility is that senescence-associated epigenetic changes extend over selected loci, as opposed to individual genes, thereby affecting the accessibility of transcription factors to linked genes within those loci. Indeed, the high mobility group box proteins, which bind non-B-type DNA, have been linked to both senescence and the SASP. HMGB1 is lost from the nuclei of senescent cells (Davalos et al., 2013), whereas HMGB2 localizes to the promoters of several SASP genes (Aird et al., 2016). This altered chromatin landscape may explain the coordinated expression of SASP genes that lie in close genomic proximity. Alternatively, as the correlated genes are regulated by similar transcription factors (such as NF-xB and C/EBPj) and likely emerged as a result of genomic duplication, it is possible that their close physical proximity allows transcription factors that leave one gene promoter for other promoters in close proximity.

An important limitation to this and similar single-cell transcription-based studies is that mRNA transcript levels may not reflect the steady-state levels of protein. Furthermore, as noted above, single-cell analyses currently score transcript levels at a single time point. Nonetheless, our analyses indicate that, at any given time, there are both uniformity in patterns and variability in individual mRNA levels in senescent cell populations.

**Experimental procedures**

**Cell culture**

IRM-90 human fetal lung fibroblasts at population doubling level 25–30 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (growth medium). Cells were seeded at 1 x 10^4 cells/cm^2 in 175-cm^2 flasks, maintained at 37°C, 10% CO_2, and 3% O_2, and were mycoplasma negative. For senescence induction, cells were given growth medium containing 50 μM bleomycin (senescent) or vehicle (PBS—nonsenescent control) in atmospheric induction for 3 hrs. The cells were then washed, given growth medium, and returned to 3% O_2. The growth medium was changed every 3 days. On day 7, after trypsinization for analysis on Day 10 (see Fig. S1A). A subset of cells was plated for senescence assays (below).

**Senescence-associated beta-galactosidase (SA-Bgal) assay**

Ten days after bleomycin or control treatment, a 12-well plate was seeded with three wells from each treatment (30 000 cells/well) and allowed to attach overnight. The assay was performed as described (Dimri et al., 1995) using a commercial kit (BioVision, Milpitas, CA, USA).

**Measurement of IL-6 secretion**

Quiescent or senescent cells were seeded as for the SA-Bgal assay and allowed to attach overnight. The following day, the medium was replaced with 500 μL of serum-free DMEM. Conditioned media were collected 24 hrs later. An ELISA kit (AlphaLISA, Perkin Elmer Biosciences, San Jose, CA, USA) was used to measure IL-6 in conditioned media according to the manufacturer’s instructions; IL-6 levels were normalized to cell number.

**EdU labeling**

Quiescent or senescent cells were plated into four-well chamber slides (Labtech, Tampa, FL, USA) and allowed to attach overnight. The next day, the cells were given low serum medium containing 10 μM EdU (Invitrogen, Carlsbad, CA, USA). After 24 hrs, cells were washed with PBS, fixed in 4% formalin, then permeabilized in 0.5% Triton X-100 for 30 min, and washed twice in ice-cold PBS. Permeabilized cells were subjected to the EdU reaction according to the manufacturer’s instructions (Click-IT EdU Imaging Kit, Invitrogen).

**Manual isolation of single cells**

Cells cultured as described above were trypsinized, pelleted by centrifugation, and resuspended in Krebs-Henseleit buffer. Approximately 20 000 cells were placed in 10 mL of buffer in a 15-cm dish. Under phase-contrast microscopy, a small glass needle was used to mouth pipette individual cells into 200-μL reaction tubes in less than 2 μL volumes. Single cells were immediately frozen on dry ice and maintained at −80°C until analysis.

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Isolation of single cells by C1

Single cells were captured in the C1 platform (Fluidigm Inc., South San Francisco, CA, USA) as per the manufacturer’s instructions (Fluidigm) and verified as single-cell captures by microscopic analysis of each nanofluidic chamber. Chambers on the C1 chip containing more than one cell were excluded from subsequent analysis.

Measurement of single-cell transcriptional profiles

Gene expression in single cells was measured by quantitative multiplex PCR following pre-amplification using the Biomark platform (Fluidigm) according to the manufacturer’s instructions. Data were normalized as described (Livak et al., 2013). Briefly, the data adjustment was as follows: Log2 expression data were extracted through the single-cell analysis package for all PCR plates, including both C1 and manual isolation data. The data were then categorized as deriving from quiescent or senescent cells prior to subsequent analyses.

Principle component analysis (PCA)

PCA was performed on qPCR values of 70 genes from 464 individual cells, 235 quiescent cells, and 229 senescent cells (one senescent cell was removed because none of its transcript was detected by qPCR) using the pcaMethods package in R (probability principle component analysis method with missing values imputed by default).

Linear discriminant analysis (LDA)

LDA was performed to maximize the separation between the 235 quiescent and 230 senescent cells. To determine which gene subsets were most important in correctly classifying cells as quiescent or senescent, we used a sequential backward selection process (Cotter et al., 2001). Starting with all the data, we used LDA to obtain the maximum separation between quiescent and senescent cells. Then, the loss of a single transcription vector was tested by removing each gene and repeating the LDA analysis with the data from the remaining 70 genes. The gene loss having the smallest impact on the ability to separate quiescent from senescent cells was deleted. This process was repeated until only the most predictive genes for discrimination remained. Because imputation of missing expression values can influence the results of LDA and the backward selection process, three separate imputation schemes were employed, with the final ranking taking each of these gene rankings into account. Missing values were first set to zero, then to the average of all values recorded for that gene, and finally to the average of all quiescent or senescent values.

Pathway analysis

Class 1 and Class 2 gene lists were submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID v.6.7) (Huang et al., 2007) for annotation enrichment analysis. The full human genome was used as a background. Pathways and annotations showing significant enrichment following Benjamini multiple testing correction are summarized in Fig. S4.

Acknowledgments

We thank Pierre-Yves Desprez for critical reading of this manuscript. This work was supported by grants from the National Institutes of Health (NIH) AG009909, AR063919 and AG017242. CW and CM were supported by NIH training grant T32-AG000266, and CW by a fellowship from the American Federation for Aging Research.

Funding

American Federation for Aging Research, (Grant / Award Number: ‘Fellowship’) National Institute on Aging, (Grant / Award Number: ‘AG009909’, ‘AG017242’, ‘T32-AG000266’) National Institute of Arthritis and Musculoskeletal and Skin Diseases, (Grant / Award Number: ‘AR063919’)

Conflict of interest

RL, JS, and MU were employed by the Fluidigm Corporation (South San Francisco) at the time C1 analyses were performed, and C1 analyses were performed at Fluidigm.

Author contributions

CW and JF designed the experiments, manually isolated single cells, and analyzed the data under the guidance of JC and SM. CW selected the genes that were analyzed, prepared senescent and quiescent cells, and performed several analyses. JF ran all qPCRs, normalized the data, and performed several analyses. CM performed LDA. XD conducted PCA with guidance from JV. RL and JS performed C1-based isolation of cells and mRNA under the guidance of MU. CW and JC wrote the article with input from SM and JV.

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