

Research Report

Cell-to-Cell Variation in Gene Expression for Cultured Human Cells Is Controlled in Trans by Diverse Genes: Implications for the Pathobiology of Aging

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Abstract

Cell-to-cell variation in gene expression increases among homologous cells within multiple tissues during aging. We call this phenomenon variegated gene expression (VGE). Long, healthy life requires robust and *coordinated* gene expression. We posit that nature may have evolved VGE as a bet-hedging mechanism to protect reproductively active populations. The price we may pay is accelerated aging. That hypothesis will require the demonstration that genetic loci are capable of modulating degrees of VGE. While loci controlling VGE in yeast and genes controlling interindividual variation in gene expression in *Caenorhabditis elegans* have been identified, there has been no compelling evidence for the role of specific genetic loci in modulations of VGE of specific targets in humans. With the assistance of a core facility, we used a customized library of siRNA constructs to screen 1,195 human genes to identify loci contributing to the control of VGE of a gene with relevance to the biology of aging. We identified approximately 50 loci controlling VGE of the longevity gene, *SIRT1*. Because of its partial homology to *FOXO3A*, a variant of which is enriched in centenarians, our laboratory independently confirmed that the knockdown of *FOXF2* greatly diminished VGE of *SIRT1* but had little impact upon the VGE of *WRN*. While the role of these VGE-altering genes on aging in vivo remains to be determined, we hypothesize that some of these genes can be targeted to increase functionality during aging.

Keywords: Variegated gene expression, Epigenetic drift, *FOXF2*, *SIRT1*, Bet hedging

Age-related drifts in gene expression among homologous somatic cell types are usually referred to as “Epigenetic Drift” (1). We are agnostic about the underlying molecular mechanisms, however, and therefore prefer the terminology of “Variegated Gene Expression” (VGE). It is indeed possible that both stochastic and programmed drifts in gene expression during the life course occur at translational and post-translational stages of gene expression, in addition to well documented transcriptional processes. VGE may have evolved as a “bet hedging” adaptation to unpredictable environments, but the widely observed increasing VGE with aging may contribute to a wide range of age-related

pathologies, when the forces of natural selection are no longer operative (2–4).

Materials and Methods

Cell Lines and Cell Culture

A human primary skin fibroblast culture 88-1 was maintained under standard culture conditions at 37°C in an atmosphere of 5% CO₂ and 5% O₂ (5). The 88-1pBlox line was generated by retroviral infection of 88-1 with excisable hTERT, pBlox-TSH,

followed by histidinol selection (6). The 88-1pBlox line was generated by retroviral infection of 88-1 with excisable hTERT, pBlox-TSH, followed by histidinol selection (6). During the siRNA screening, cell lines were kept in the 5% CO₂ incubator with ambient oxygen (7).

Establishment of an Inducible *FOXF2* shRNA Cell Line

Commercially obtained lentiviral *FOXF2* shRNAs were introduced to 88-1pBlox following the manufacturer's instructions (Dharmacon V3SH7675; <https://dharmacon.horizondiscovery.com/uploadedFiles/Resources/smartvector-inducible-lentiviral-shRNA-manual.pdf>). Doxycycline (DOX) concentration was titrated to determine the minimum dosage (3 μM) with maximum GFP induction coded in the lentiviral construct (8).

siRNA Library Screening

A customized siRNA Epigenetics library was chosen that included a wide range of loci of potential relevance to epigenetics. Screening of 1,195 loci was performed as previously described (7). Two hundred 88-1pBlox cells were plated per well in 384-well plates. Twenty-four hours later, cells were transfected with the siRNA library using DharmaFECT1 (T-2001, Dharmacon, Lafayette, CO). Seventy-two hours later, plates were fixed and stained with rabbit anti-SIRT1 antibodies (1:100, PA5-17232, Thermo Fisher, Waltham, MA), followed

by secondary antibody staining using donkey anti-rabbit IgG conjugated with Alexa 488 (1:1,500, A-21206, Thermo Fisher). Nuclei were counter-stained with Hoechst 33342 (62249, Thermo Fisher). Alexa 488 intensity overlapping Hoechst 33342 was measured with IN Cell Analyzer 2000 (Quellos High Throughput Screening Core) to determine relative per-cell expression of SIRT1 protein. Ranges of nuclear SIRT1 protein expression in each well were divided by the population range of SIRT1 to calculate the fold changes of VGE.

RNA Extraction and Quantitative RT-PCR

The *FOXF2* transfected cultures were split into two sets. One set was treated with doxycycline (DOX+) to induce shRNA expression, the other with standard fibroblast medium (DOX-) to serve as the control. Total RNA was isolated from the cultured cell pellet with TRIzol reagent (15596018, Thermo Fisher) following manufacturer's instructions. Reverse transcription was carried out in a 40 μL reaction with 2 μg of isolated total RNA. Quantitative RT-PCR was performed as previously described (9). Quantitative RT-PCR for *FOXF2*, *p16*, and *p21* was performed using TaqMan Gene Expression Assay System (Hs00230963_m1, Hs00923894_m1, and Hs99999142_m1 (Applied Biosystem, Foster City, CA) and normalized against the mRNA levels of GAPDH (Hs02758991_g1, Applied Biosystem). The reaction was run in a QuantStudio3 real-time PCR system (Applied Biosystem).

Table 1. Changes of the Degrees of Variegated Gene Expression Following siRNA Introduction in Human Fibroblasts, 88-1pBlox

A. siRNA library screening for loci that modulate cell-to-cell variation. Loci whose siRNAs altered variegation of *SIRT1* expression are sorted based on the fold changes of the range of *SIRT1* expression relative to controls. The rankings are listed from lowest to highest levels of VGE. *FOXF2* was chosen as an initial target of interest for subsequent validations.

Rank	Gene Locus	Gene Name	Cellular Function	Fold Change of Range	<i>p</i> Value
1	<i>SUPV3L1</i>	Suv3 like RNA helicase	Suppressor of Var-1 mutant. Mitochondrial helicase	-10.54	1.03E-14
2	<i>SMPD2</i>	Sphingomyelin phosphodiesterase 2, neutral membrane	Lysophospholipase	-9.15	3.56E-10
3	<i>FOXF2</i>	Forkhead box F2	Partial homology to FOX3A transcription factor	-6.92	1.34E-06
23	<i>EP400</i>	E1A binding protein p400	Senescence-associated chromatin regulator	-2.65	.01
25	<i>FOXS1</i>	Forkhead box S1	Partial homology to FOX3A transcription factor	-2.50	.024
1190	<i>WHSC1</i>	Histone-Lysine N-Methyltransferase NSD2	Histone methyltransferase	5.69	7.36E-04
1193	<i>UTY</i>	Ubiquitously transcribed tetrapeptide repeat gene	Y-linked minor histocompatibility antigen	6.95	6.31E-05
1194	<i>TIMM50</i>	Translocase of inner mitochondrial membrane 50 homolog	Inner mitochondrial membrane translocase complex.	9.36	1.51E-07
1195	<i>TH</i>	Tyrosine hydroxylase	Conversion of tyrosine to dopamine	12.65	1.85E-13

B. Confirmation of reduced cell-to-cell variation of *SIRT1*, but not of *WRN* expression, following the introduction of Doxycycline (DOX) inducible *FOXF2* siRNA in 88-1pBlox.

	SIRT1 Experiment 1		SIRT1 Experiment 2		WRN Experiment 1		WRN Experiment 2	
	DOX-	DOX+	DOX-	DOX+	DOX-	DOX+	DOX-	DOX+
Cell number	345	304	410	519	484	689	383	521
Mean	27.70	16.30	18.44	13.24	31.17	29.08	29.80	29.58
SD	6.43	2.75	4.82	2.42	5.40	4.86	6.93	7.28
Coefficients of variation	23.21	16.85	26.16	18.26	17.33	16.72	23.25	24.60
<i>F</i> -test <i>p</i> value	5.10E-46		3.17E-48		.026		.25	

The levels of transcript knockdown were calculated with comparative C_T value method (9).

Indirect Immunostaining

The immunostaining of the 88-1pBlox shFOXF2 cells was performed as previously described (10). The antibodies used were rabbit anti-SIRT1 antibodies (1:100, PA5-17232, Thermo Fisher), and donkey anti-rabbit IgG conjugated with Alexa 594 (1:200, A-21207, Thermo Fisher). Stained cells were imaged with an EVOS FL microscope (Thermo Fisher). The images were converted to 12 bit TIFF files and the stained nuclei were traced and boxed with a graphing tablet in order to measure the mean intensity within each boxed nucleus. For each set of stains, duplicate assays of approximately 400 nuclei were traced using a graphing tablet for measurements of mean intensities of foci (10). The means and standard deviations of each data set were calculated to compute the coefficients of variation. The results were analyzed using Excel to obtain the coefficient of variation for each of the stained cell lines.

Statistical Analysis

Statistical significance was determined by the Student's *t* test.

Results

A key prediction of this hypothesis of VGE as an antagonistic pleiotropic mechanism of aging is that the magnitude of VGE should be under genetic control. This was the underlying motivation for the present line of research. Therefore, we performed a high throughput screening experiment at the Quellos High Throughput Screening Core (<https://iscrm.uw.edu/research/core-resources/quellos-high-throughput-screening-core/>) utilizing a siRNA library targeting 1,195 loci (7) (Supplementary Figure S1). We identified several noteworthy loci that modulated the degree of variation of *SIRT1* expression in 88-1pBlox, an immortalized human diploid fibroblast cell line (Table 1, A). *TH*, *TIMM50*, and *UTY* were the top genes acting to reduce-cell-to-cell variation in gene expression (their knockdowns resulted in increased cell-to-cell variation of *SIRT1*). On the other side of the spectrum, *SUPV3L1*, *SMPD2*, and *FOXOF2* were the top three genes promoting VGE (Table 1, A). We selected forkhead transcription factor *FOXF2* for further validation, given the roles that forkhead transcription factor FOXO3 has in the longevity of human centenarians (11). We were also motivated to pursue these studies because of prior research by our coauthors on decreasing VGE (interindividual variation) in *C elegans* (12) and prior research in yeast, showing that genes can control the cell to cell variation in the expression of other gene (13,14).

To validate our findings, we transfected the derived 88-1pBlox immortalized human diploid fibroblast cell line used for the initial screening with three distinct inducible *FOXF2* shRNA constructs, each targeting distinct transcript sequences. We chose the shRNA construct that produced the most robust knockdown effect (9 fold) for *FOXF2* (Figure 1) for subsequent experiments. We quantified two nuclear proteins, *SIRT1* and *WRN* (the latter not included in the Epigenetics library), using immunohistochemistry to compare gene expression levels in cells. *SIRT1* staining data agreed with the high throughput screening results. We found that *FOXF2* knockdown reproducibly decreased cell-to-cell variation in *SIRT1* expression. *FOXF2* knockdown did not reproducibly alter cell-to-cell variation in *WRN* expression (Table 1, B; Supplementary Figure

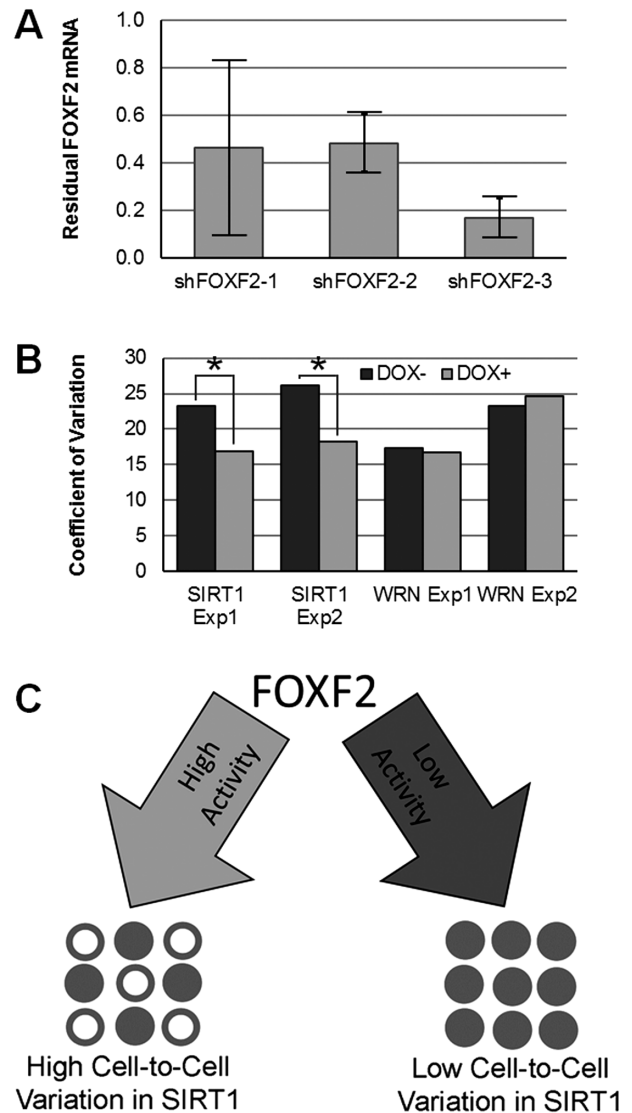


Figure 1. Demonstration of the *FOXF2*-mediated modulation of variegated gene expression of *SIRT1*. (A) Quantitative RT-PCR of *FOXF2* mRNA in 88-1pBlox cell line with 3 different *FOXF2* shRNAs. Relative *FOXF2* mRNA after *FOXF2* shRNA expression was induced by doxycycline. (B) Reduction of cell-to-cell variation of *SIRT1* expression, but not in *WRN* expression, in 88-1pBlox + shFOXF2-3. * indicates statistical significance. (C) A schematic overview of the *FOXF2*-mediated VGE.

S2). In vivo effects of *FOXF2* on cell-to-cell variation and life span remain to be tested.

Discussion

Rising levels of VGE may contribute to the pathogenesis of a wide variety of geriatric disorders (4). We also note that increasing VGE may not happen for all genes in all tissues, as some recent data suggests that some genes in some cell types may not alter or may decrease VGE with age (15). However, the discovery of genetic loci capable of modulating the degrees of VGE may have important translational applications for the extensions of human health span and life span. Consider, for example, the results of increased degrees of aberrant expressions of the subsets of genetic

loci responsible for proliferative homeostasis. One can envision insufficient rates of replacement of somatic cells, leading to tissue atrophy. Conversely, one can also envision multifocal regions of hyperplasia. Both of these phenotypes are regularly observed in autopsies of aging mice and humans. The accelerated rates of DNA synthesis associated with hyperplasias can be expected to lead to increasing probabilities of mutations in oncogenes and tumor suppressor genes, leading to neoplasias. The role of epigenetic drift in cancer progression has been well posited and discussed by Feinberg and colleagues (13,14,16).

The discovery of loci with the potential to modulate degrees of VGE now opens the door to tests of the hypothesis that decreases in the age-related increases of VGE will lead to ameliorations of multiple features of the pathobiology of aging via the genetic engineering of strains of experimental organisms, notably using well characterized strains of laboratory mice and of *C elegans*.

Supplementary Material

Supplementary data is available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

No conflicts of interest declared.

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