THE GERONTOLOGICAL SOCIETY OF AMERICA®

*Journals of Gerontology: Biological Sciences*
cite as: *J Gerontol A Biol Sci Med Sci*, 2020, Vol. XX, No. XX, 1–4
doi:10.1093/gerona/glaa027
Advance Access publication January 20, 2020

OXFORD



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

**Jiaming Zhang, BS,[1] Nikolay Burnaevskiy, PhD,[1] James Annis, BS, MBA,[2] Wenyan Han, BS,[1] Deyin Hou,[1] Paula Ladd, PhD,[1] Lin Lee, BS,[1] Alexander R. Mendenhall, PhD,[1] Junko Oshima, MD, PhD,[1] and George M. Martin, MD[1,*]**

[1]Department of Pathology, University of Washington, Seattle. [2]Quellos High-throughput Screening Core, Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle.

*Address correspondence to: George M. Martin, MD, Department of Pathology, University of Washington, Box 357470, HSB K-543, Seattle, WA, 98195. E-mail: gmmartin@uw.edu

Received: October 17, 2019; Editorial Decision Date: January 9, 2020

**Decision Editor:** David Le Couteur, MBBS, FRACP, PhD

## 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 prolongevity 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% $CO_2$ and 5% $O_2$ (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 (Dhamracon 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 p21was 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 | *p* Value |
|---|---|---|---|---|---|
| 1 | *SUPV3L1* | Suv3 like RNA helicase | Suppressor of Var-1 mutant. Mitochondrial helicase | −10.54 | 1.03E−14 |
| 2 | *SMPD2* | Sphingomyelin phosphodiesterase 2, neutral membrane | Lysophospholipase | −9.15 | 3.56E−10 |
| 3 | ***FOXF2*** | **Forkhead box F2** | **Partial homology to FOX3A transcription factor** | −6.92 | 1.34E−06 |
| 23 | *EP400* | E1A binding protein p400 | Senescence-associated chromatin regulator | −2.65 | .01 |
| 25 | *FOXS1* | Forkhead box S1 | Partial homology to FOX3A transcription factor | −2.50 | .024 |
| 1190 | *WHSC1* | Histone-Lysine N-Methyltransferase NSD2 | Histone methyltransferase | 5.69 | 7.36E−04 |
| 1193 | *UTY* | Ubiquitously transcribed tetratricopeptide repeat gene | Y-linked minor histocompatibility antigen | 6.95 | 6.31E−05 |
| 1194 | *TIMM50* | Translocase of inner mitochondrial membrane 50 homolog | Inner mitochondrial membrane translocase complex. | 9.36 | 1.51E−07 |
| 1195 | *TH* | 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 Doxycyclin (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 |
| *F*-test *p* 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 S2). In vivo effects of FOXF2 on cell-to-cell variation and life span remain to be tested.

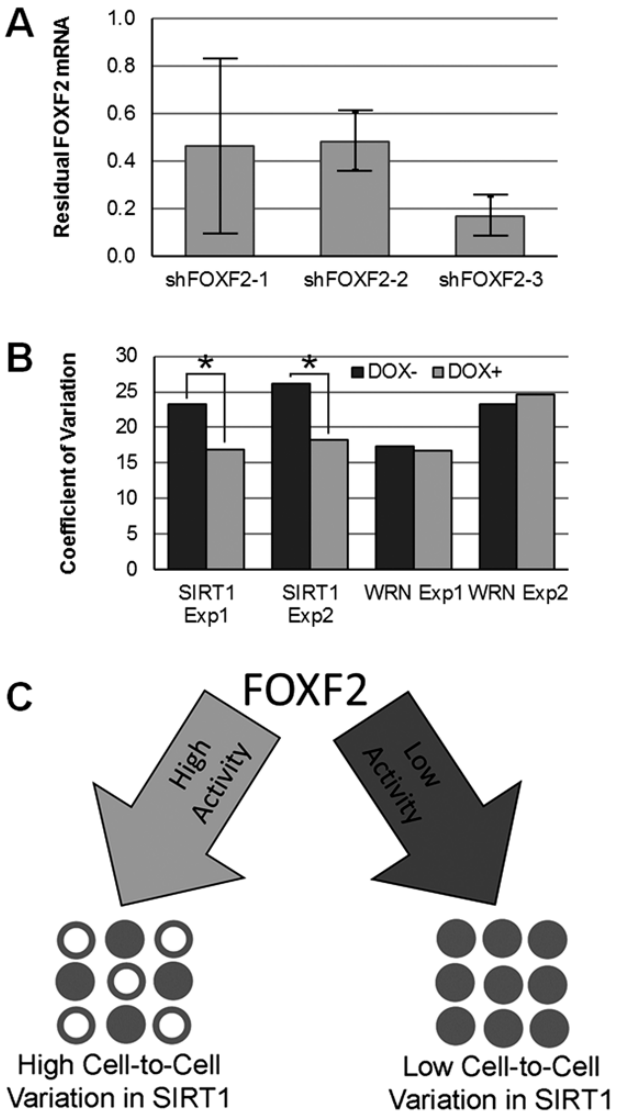


**Figure 1.** Demonstration of the *FOXF2*-mediated modulation of variegated gene expression of *SIRT1*. (**A**) Quantitative RT-PCR of FOXF2 mRNA in 88-1pBlox cell line with 3 different *FOXF2* shRNAs. Relative FOXF2 mRNA after *FOXF2* shRNA expression was induced by doxycycline. (**B**) Reduction of 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.

## 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.

## Funding

This work was supported in part by National Institute of Health (R00AG045341 and R01CA219460 to A.M., K99AG061216 to N.B., R01CA210916 to G.M.M./J.O.); and Japan Society for the Promotion of Science (KAKENHI 17H04037 to J.O.)

## Acknowledgments

J.Z., W.H., D.H., P.L., and L.L. established the cell lines and conducted molecular and cell biological experiments; N.B. developed and conducted imaging analyses; J.A. conducted siRNA screening; A.R.M., J.O., and G.M.M. designed the study and wrote the manuscript. We thank Mr. Binyamin Levin for editorial assistance.

## Conflict of Interest

No conflicts of interest declared.

## References

1. Issa JP. Aging and epigenetic drift: a vicious cycle. *J Clin Invest*. 2014;124:24–29. doi: 10.1172/JCI69735
2. Bahar R, Hartmann CH, Rodriguez KA, et al. Increased cell-to-cell variation in gene expression in ageing mouse heart. *Nature*. 2006;441:1011–1014. doi: 10.1038/nature04844
3. Martin GM. Epigenetic gambling and epigenetic drift as an antagonistic pleiotropic mechanism of aging. *Aging Cell*. 2009;8:761–764. doi: 10.1111/j.1474-9726.2009.00515.x
4. Martin GM. Stochastic modulations of the pace and patterns of ageing: impacts on quasi-stochastic distributions of multiple geriatric pathologies. *Mech Ageing Dev*. 2012;133:107–111. doi: 10.1016/j.mad.2011.09.001
5. Oshima J, Campisi J, Tannock TC, Martin GM. Regulation of c-fos expression in senescing Werner syndrome fibroblasts differs from that observed in senescing fibroblasts from normal donors. *J Cell Physiol*. 1995;162:277–283. doi: 10.1002/jcp.1041620213
6. Rubio M, Kim S, Campisi J. Reversible manipulation of telomerase expression and telomere length. *J Biol Chem*. 2002;277:28609–28617. doi:10.1074/jbc.m203747200
7. Zhang J, Hou D, Annis J et al. Inactivating mutations in exonuclease and polymerase domains in DNA polymerase delta alter sensitivities to inhibitors of dNTP synthesis. *DNA Cell Biol*. 2020;39:50–56. doi: 10.1089/dna.2019.5125
8. Loew R, Heinz N, Hampf M, Bujard H, Gossen M. Improved tet-responsive promoters with minimized background expression. *BMC Biotechnol*. 2010;10:81. doi: 10.1186/1472-6750-10-81
9. Saha B, Zitnik G, Johnson S, et al. DNA damage accumulation and TRF2 degradation in atypical Werner syndrome fibroblasts with LMNA mutations. *Front Genet*. 2013;4:129. doi:10.3389/fgene.2013.00129
10. Sargolzaeiaval F, Zhang J, Schleit J, et al. CTC1 mutations in a Brazilian family with progeroid features and recurrent bone fractures. *Mol Genet Genomic Med*. 2018;6:1148–1156. doi: 10.1002/mgg3.495
11. Revelas M, Thalamuthu A, Oldmeadow C, et al. Review and meta-analysis of genetic polymorphisms associated with exceptional human longevity. *Mech Ageing Dev*. 2018;175:24–34. doi: 10.1016/j.mad.2018.06.002
12. Mendenhall A, Crane MM, Tedesco PM, Johnson TE, Brent R. *Caenorhabditis elegans* genes affecting interindividual variation in life-span biomarker gene expression. *J Gerontol A Biol Sci Med Sci*. 2017;72:1305–1310. doi: 10.1093/gerona/glw349
13. Feinberg AP, Koldobskiy MA, Göndör A. Epigenetic modulators, modifiers and mediators in cancer aetiology and progression. *Nat Rev Genet*. 2016;17(5):284–299. doi:10.1038/nrg.2016.13
14. Timp W, Feinberg AP. Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host. *Nat Rev Cancer*. 2013;13:497–510. doi: 10.1038/nrc3486
15. Ximerakis M, Lipnick SL, Innes BT, et al. Single-cell transcriptomic profiling of the aging mouse brain. *Nat Neurosci*. 2019;22:1696–1708. doi: 10.1038/s41593-019-0491-3
16. Feinberg AP. Epigenetic stochasticity, nuclear structure and cancer: the implications for medicine. *J Intern Med*. 2014;276:5–11. doi: 10.1111/joim.12224