



Cell division rates decrease with age, providing a potential explanation for the age-dependent deceleration in cancer incidence

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Contributed by Bert Vogelstein, July 15, 2019 (sent for review April 4, 2019; reviewed by Ronny Drapkin and Michel Wassef)

A new evaluation of previously published data suggested to us that the accumulation of mutations might slow, rather than increase, as individuals age. To explain this unexpected finding, we hypothesized that normal stem cell division rates might decrease as we age. To test this hypothesis, we evaluated cell division rates in the epithelium of human colonic, duodenal, esophageal, and posterior ethmoid sinonasal tissues. In all 4 tissues, there was a significant decrease in cell division rates with age. In contrast, cell division rates did not decrease in the colon of aged mice, and only small decreases were observed in their small intestine or esophagus. These results have important implications for understanding the relationship between normal stem cells, aging, and cancer. Moreover, they provide a plausible explanation for the enigmatic age-dependent deceleration in cancer incidence in very old humans but not in mice.

cell division | aging | cancer | mutation rate

Somatic mutation rates have been measured in several human cell types with a variety of techniques (1–5). For both nuclear and mitochondrial genomes, somatic mutations appear to increase in a linear fashion throughout life. On the surface, this accumulation is consistent with the increase in tumor incidence with age that forms one of the cornerstones of our understanding of cancer (6, 7). This relationship between age and cancer incidence has important practical and conceptual ramifications, given that the proportion of very old individuals in the population is expected to increase. As noted by many investigators, however, there is a blemish in this otherwise satisfying picture: In very old individuals, cancer incidence rates often decelerate (8–11). Although hypotheses for this deceleration have been proposed, understanding of the deceleration of cancer incidence with age remains incomplete (8, 10–13). This conundrum led us to question whether the expected linear accumulation of mutations with age continues throughout life, even in old individuals. While evaluating the literature on this topic (4, 14, 15), we noted that it was generally assumed, rather than demonstrated, that the mutations continued to accumulate in a linear fashion over time. When we removed the assumption of linearity from such regression analyses, a different picture often seemed to emerge: a trend toward slowing in the rate at which somatic mutations accumulate with age (*SI Appendix, Figs. S1–S3*). This observation is supported by the analyses provided by Podolskiy et al. (16)

How could this occur? It is unlikely that the DNA polymerases responsible for replicating DNA acquire increased fidelity or

that DNA repair processes improve in aged individuals, and no evidence for such beneficial changes in repair has emerged. We considered it more likely that cell division rates decrease with age. There is little known about division of stem cells in human self-renewing tissues over time except that the number (rather than division rate) of hematopoietic stem cells and epidermal stem cells appear not to decrease with age (17, 18).

Results

Cell Division Rates Decelerate in Human Tissues. To test the hypothesis that cell division rates may actually decrease with age, histologically normal colon samples were obtained from 13 individuals in each of 2 age cohorts: 20 to 29 y of age and 80 to 89 y of age. We used the Ki67 antibody, a well-established proliferation marker, to label sections from these tissues (*Materials and Methods*). Ki67 labels nuclei during all active phases of the

Significance

We have discovered a species-specific feature of the aging process: significantly slowed division rates in self-renewing tissues of humans. In contrast, we found only small to no decrease in division rates in self-renewing tissues of mice, for whom cancer incidence does not decelerate with age. This discovery has important implications for the process of aging itself, particularly given that there is so little known about this ubiquitous process. It additionally provides a potential explanation, supported by experimental data, for the enigmatic decrease of cancer in the oldest (and most rapidly growing) segment of Western populations.

Author contributions: C.T. conceived the study; C.T., C.A.I.-D., and B.V. designed research; C.T., J.P., N.J.R., N.R.L., M.E.P., M.C.H., A.R., A.B., B.K., A.K., C.M.H., A.K.M., R.H.H., C.A.I.-D., and B.V. performed research; C.T., C.M.H., and A.K.M. contributed new reagents/analytic tools; C.T. and B.V. analyzed data; and C.T., J.P., N.J.R., N.R.L., M.E.P., M.C.H., A.B., C.M.H., A.K.M., R.H.H., C.A.I.-D., and B.V. wrote the paper.

Reviewers: R.D., University of Pennsylvania; and M.W., Institut Curie, Paris Sciences et Lettres Research University.

The authors declare no conflict of interest.

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Data deposition: All data are available on GitHub (<https://github.com/cristomasetti/>).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1905722116/-DCSupplemental.

First Published September 23, 2019.

cell cycle (G_1 , S , G_2 , and mitosis), but is absent from resting cells (G_0).

We identified 151 labeled cells (15% of the 992 analyzed cells) in the older cohort at positions 1 to 8 from the crypt bases—the region where the stem cells are thought to reside, whereas in the younger cohort, 219 labeled cells were found (26% of the 848 analyzed cells) (Fig. 1). The difference in Ki67 labeling was highly significant ($P < 3 \cdot 10^{-3}$), with the proliferation rate in the older group reduced by 41% of that in the younger group (95% confidence interval [CI] = 23 to 59%) (experiment 1A, Fig. 2).

Moreover, a similar pattern was observed throughout the replicating portion of the colonic epithelium (Dataset S1, referring to experiment 1A).

A reanalysis performed by a second pathologist, using an automated imaging method for the counting of labeling in the same samples along the full crypt, confirmed these results: a highly significant 44% reduction ($P < 5 \cdot 10^{-7}$) in the older group (95% CI = 28 to 60%) (experiment 1B, Fig. 2 and Materials and Methods).

To further validate these findings, experiments were performed on 2 independent sets of histologically normal human colon tissues using the same labeling technique (experiments 2 and 3, Fig. 2). In experiment 3, an antibody that recognizes the lamin A/C protein was utilized to better visualize the outline of each nucleus, particularly the regions that have high cellularity (Materials and Methods and SI Appendix, Fig. S4). Again, a significant decrease in cell proliferation at the first 8 positions from the crypt basis was found in the older cohorts in both experiments: a 37% (95% CI = 21 to 52%; $P < 9 \cdot 10^{-6}$) reduction and

a 39% (95% CI = 13 to 65%; $P < 4 \cdot 10^{-3}$) reduction, respectively. These results, highly significant even when controlling for multiple testing, document that colonic crypt epithelial cell proliferation is reduced in older individuals.

We also performed a fourth experiment where the colonic tissues were double-stained for Ki67 as well as Lgr5, a marker associated with colonic stem cells. The double-positive cells were too few to obtain a statistically significant result, but a trend was found for the younger colons having higher proliferation than the older cohort, with 16 double-stained cells (out of 14,400 total cells counted [i.e., 0.001%]) in the old cohort vs. 56 double-stained cells (out of 18,416 total cells counted [i.e., 0.003%]) in the young cohort (Materials and Methods and SI Appendix, Fig. S5).

Finally, in colon, we performed a fifth experiment on 46 paraffin-embedded sections of histologically normal colon (20 samples in the younger cohort and 26 in the older cohort), where the colonic tissues were labeled using an antibody raised against the mitosis-specific phosphorylation of histone H3 protein (pHH3); this protein identifies dividing cells within the late G_2 phase to M phase of the cell cycle (19), and represents an alternative marker of cell proliferation. By analyzing the first 8 cell positions from the base of the crypts on each side, we identified 14 labeled cells (1.02% of 1,376 cells analyzed) in the younger cohort, and we identified 10 labeled cells (0.53% of the 1,872 cells analyzed) in the older cohort, using 2 to 7 widely separated sections of tissue from each case (Materials and Methods and SI Appendix, Fig. S6). Although these differences did not reach statistical significance due to the very low number of positive cells ($P = 0.084$), they suggest a 48% decrease in labeling in the older cohort compared with the younger cohort, again supporting our findings using Ki67.

We next evaluated histologically normal human esophageal biopsies from another 20 individuals in each age cohort (Materials and Methods).

The esophagus is lined by nonkeratinizing squamous epithelium; therefore, the organization of cells in the esophagus differs from that in the intestines. Thus, a slightly different strategy was used to assess the proportion of labeled cells in this tissue type. The stem cells in the esophagus are confined to the basal layer, so only the basal layer was analyzed, and sections were chosen in which the basal layer was consistently oriented with respect to the long axes of the embedded tissue (Fig. 3). A mean of 54% of the basal layer cells was labeled with Ki67 in the younger cohort compared with 44% in the older cohort. Thus, the older cohort group had a significant 19% decrease (95% CI = 12 to 25%; $P < 9 \cdot 10^{-8}$) in proliferation activity compared with the younger group (experiment 1A, Fig. 4).

Using an automated imaging method, a second pathologist reanalyzed these tissues, without restricting the analysis to the basal layer. A mean of 33% and 52% labeled cells per 40 \times field were identified as positive in the older and younger cohorts, respectively. Thus, the older cohort group had a significant 37% decrease (95% CI = 29 to 45%; $P < 2 \cdot 10^{-14}$) of the proliferation activity of the younger group (experiment 1B, Fig. 4 and Materials and Methods).

To further validate these findings, experiments were performed on 2 independent sets of human esophageal samples using the same labeling technique (experiments 2 and 3, Fig. 4). In experiment 3, an antibody that recognizes the lamin A/C protein was utilized to better visualize the outline of each nucleus, particularly the regions that have high cellularity (Material and Methods). Again, a significant decrease in cell proliferation in the basal layer of the esophagus was found in the older cohorts in both experiments: a 10% (95% CI = 4 to 16%; $P < 8 \cdot 10^{-4}$) decrease and 31% (95% CI = 8 to 53%; $P < 8 \cdot 10^{-3}$) decrease, respectively. These results remain significant even when controlling

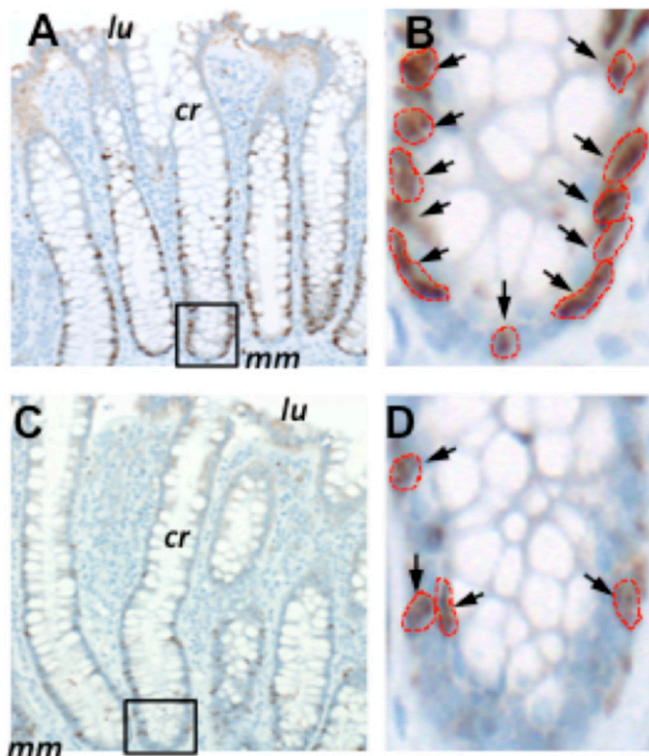


Fig. 1. Ki67 labeling of normal colon resections. Shown are examples of Ki67 labeling in a 26-y-old individual (A and B) compared with an 80-y-old individual (C and D). *cr*, properly oriented crypt; *lu*, location of the colonic lumen; *mm*, muscularis mucosae. The crypts outlined in A and C are magnified in B and D, respectively. Arrows indicate antibody-labeled nuclei, which are outlined in red. Images in A and C were taken at a magnification of 100 \times , while those in B and D were taken at a magnification of 400 \times .

P3563-10PAK; Sigma Life Sciences). Slides were then incubated in blocking solution (catalog no. S2003; Dako) for 5 min. Ki67 primary antibody (catalog no. Ki67P-CE; Leica) was diluted 1:500 in Antibody Dilution Buffer (catalog no. ADB250; Ventana) before staining slides for 45 min at room temperature. After washing, slides were incubated with a secondary antibody (catalog no. PV6119; Leica) for 30 min at room temperature. They were then incubated with DAB (3,3'-diaminobenzidine) reagent (catalog no. D4293-50SET; Sigma Life Sciences) and counterstained with hematoxylin. Ten regions of esophagus, small intestine, and colon were randomly chosen from the slides of each mouse. The nuclei within these regions were assessed for Ki67 positivity, counting the basal layer of the esophagus or the crypt cells (positions 1 to 8) of the intestines, using well-oriented crypts as described above (Fig. 8).

Scoring Pathologist Information. The slides were scored by the following pathologists: colon experiment 1A (J.P.), colon experiment 1B (A.B.), colon experiment 2 (C.A.I.-D.), and colon experiment 3 (C.A.I.-D.); esophagus experiment 1A (M.E.P.), esophagus experiment 1B (A.B.), esophagus experiment 2 (M.E.P.), and esophagus experiment 3 (C.A.I.-D.); duodenum (J.P.); posterior ethmoid (M.C.H.); colon Ki67 and Lgr5 (M.E.P.); colon Ki67 and lamin A/C (C.A.I.-D.); colon pHH3 (J.P.); and all countings on mice samples (J.P. and M.E.P.).

All datasets are provided in [Datasets S1–S11](#).

Statistical Analysis of Ki67 Labeling. The significance of the differences in the number of positive cells among all tissue samples from humans, as well as for all tissues from mice, was determined by the Welch *t* test, providing an estimate for the difference in the means between the 2 groups and the CI. All statistical analyses were performed using R software, version 3.5.1 (R Development Core Team).

Previous Literature on the Effect of Aging on Cell Proliferation. There have been a few attempts in the past to estimate the effect of aging on cell proliferation, and we comment briefly on them.

Corazza et al. (26) used proliferating cell nuclear antigen (PCNA), which we do not consider as good as Ki67 as a cell division marker because PCNA is important for both DNA synthesis and DNA repair (DNA polymerase

epsilon is involved in resynthesis of excised damaged DNA strands during DNA repair).

Ciccocioppo et al. (25) used MIB-1 and showed an increase of apoptosis as well as an increase in cell proliferation in older people in the crypt, in contrast to our findings, but not in the villi. They state: "It should be underlined that, as far as MIB-1 expression is concerned, the present results are slightly at variance from those of a previous study of ours in which a different marker of enterocyte proliferation (PCNA) was used [Corazza et al. (26)]. Both PCNA and MIB-1 expression was increased in aged crypt enterocytes, but only PCNA turned out to be raised at the villous level."

Roncucci et al. (27) used [³H]thymidine in rectal mucosa and concluded that proliferation is higher in older people. However, when considering the 5 compartments in which the crypts have been divided (with 1 being the lowest, the base of the crypt [figure 1 in ref. 27]), we find 2 results consistent with ours and 1 that is not: (1) The lowest compartment (base of the crypt) had a higher proliferation than the higher compartments, independent of age, precisely as we show in our [Dataset S2](#), and (2) the lowest compartment 1 had more proliferation in young patients than in old patients in agreement with our results and contrary to their overall claim; thus, in the stem cell range, their results agree with ours. (3) In the upper compartment, however, they find more proliferation in old than young patients. Here, we think that since the signal, as demonstrated in point 1 by both us and them, gets smaller and smaller in the upper compartments, noise is probably confounding the results. We cannot explain, however, the difference with our results in the intermediate compartments (II and III).

Potential limitations of all these studies are a lack of technical and biological experiment replicates, a small number of slides and crypts analyzed per sample (not reported), and the fact that only 1 tissue was considered.

ACKNOWLEDGMENTS. We thank David L. Huso, who tragically passed away during the preparation of this manuscript, for his contribution to the experiments. This work was supported by The John Templeton Foundation; The Maryland Cigarette Restitution Fund; The Virginia and D. K. Ludwig Fund for Cancer Research; The Lustgarten Foundation for Pancreatic Cancer Research; The Sol Goldman Pancreatic Cancer Research Center; and NIH Grants P30-CA006973, R37-CA43460, R01-CA57345, R01-CA179991, R00-CA190889, and P50-CA62924.

- D. J. Araten et al., A quantitative measurement of the human somatic mutation rate. *Cancer Res.* **65**, 8111–8117 (2005).
- R. DeMars, K. R. Held, The spontaneous azaguanine-resistant mutants of diploid human fibroblasts. *Humangenetik* **16**, 87–110 (1972).
- J. W. Drake, B. Charlesworth, D. Charlesworth, J. F. Crow, Rates of spontaneous mutation. *Genetics* **148**, 1667–1686 (1998).
- C. Tomasetti, B. Vogelstein, G. Parmigiani, Half or more of the somatic mutations in cancers of self-renewing tissues originate prior to tumor initiation. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 1999–2004 (2013).
- J. S. Welch et al., The origin and evolution of mutations in acute myeloid leukemia. *Cell* **150**, 264–278 (2012).
- R. Armitage, R. Doll, The age distribution of cancer and a multi-stage theory of carcinogenesis. *Br. J. Cancer* **8**, 1–12 (1954).
- C. O. Nordling, A new theory on cancer-inducing mechanism. *Br. J. Cancer* **7**, 68–72 (1953).
- P. R. Burch, Natural and radiation carcinogenesis in man. I. Theory of initiation phase. *Proc. R. Soc. Lond. B Biol. Sci.* **162**, 223–239 (1965).
- S. A. Frank, *Dynamics of Cancer: Incidence Inheritance, and Evolution* (Princeton University Press, Princeton, NJ, 2007).
- H. A. Hanson, K. R. Smith, A. M. Stroup, C. J. Harrell, An age-period-cohort analysis of cancer incidence among the oldest old, Utah 1973–2002. *Popul. Stud. (Camb.)* **69**, 7–22 (2015).
- S. Horiuchi, J. R. Wilmoth, Deceleration in the age pattern of mortality at older ages. *Demography* **35**, 391–412 (1998).
- P. J. Cook, R. Doll, S. A. Fellingham, A mathematical model for the age distribution of cancer in man. *Int. J. Cancer* **4**, 93–112 (1969).
- J. W. Vaupel et al., Biodemographic trajectories of longevity. *Science* **280**, 855–860 (1998).
- R. J. Albertini, J. A. Nicklas, J. P. O'Neill, S. H. Robison, In vivo somatic mutations in humans: Measurement and analysis. *Annu. Rev. Genet.* **24**, 305–326 (1990).
- J. Cole, T. R. Skopek, International Commission for Protection Against Environmental Mutagens and Carcinogens. Working paper no. 3. Somatic mutant frequency, mutation rates and mutational spectra in the human population in vivo. *Mutat. Res.* **304**, 33–105 (1994).
- D. I. Podolskiy, A. V. Lobanov, G. V. Kryukov, V. N. Gladyshev, Analysis of cancer genomes reveals basic features of human aging and its role in cancer development. *Nat. Commun.* **7**, 12157 (2016).
- H. Raveh-Amit, S. Berszenyi, V. Vas, D. Ye, A. Dinnyes, Tissue resident stem cells: Till death do us part. *Biogerontology* **14**, 573–590 (2013).
- A. Giangreco, M. Qin, J. E. Pintar, F. M. Watt, Epidermal stem cells are retained in vivo throughout skin aging. *Aging Cell* **7**, 250–259 (2008).
- M. J. Hendzel et al., Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348–360 (1997).
- C. Harding, F. Pompei, R. Wilson, Corrections to: "Age distribution of cancer in mice". *Toxicol. Ind. Health* **27**, 265–270 (2011).
- G. P. Crossan, J. I. Garaycochea, K. J. Patel, Do mutational dynamics in stem cells explain the origin of common cancers? *Cell Stem Cell* **16**, 111–112 (2015).
- C. Tomasetti, B. Vogelstein, Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science* **347**, 78–81 (2015).
- M. C. Florian et al., A canonical to non-canonical Wnt signalling switch in haematopoietic stem-cell ageing. *Nature* **503**, 392–396 (2013).
- M. Sinha et al., Restoring systemic GDF11 levels reverses age-related dysfunction in mouse skeletal muscle. *Science* **344**, 649–652 (2014).
- R. Ciccocioppo et al., Small bowel enterocyte apoptosis and proliferation are increased in the elderly. *Gerontology* **48**, 204–208 (2002).
- G. R. Corazza et al., Proliferating cell nuclear antigen expression is increased in small bowel epithelium in the elderly. *Mech. Ageing Dev.* **104**, 1–9 (1998).
- L. Roncucci et al., The influence of age on colonic epithelial cell proliferation. *Cancer* **62**, 2373–2377 (1988).
- C. Tomasetti et al., Role of stem-cell divisions in cancer risk. *Nature* **548**, E13–E14 (2017).
- C. Tomasetti, L. Li, B. Vogelstein, Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. *Science* **355**, 1330–1334 (2017).
- C. Tomasetti, B. Vogelstein, Cancer risk: Role of environment—Response. *Science* **347**, 729–731 (2015).
- C. Tomasetti, B. Vogelstein, On the slope of the regression between stem cell divisions and cancer risk, and the lack of correlation between stem cell divisions and environmental factors-associated cancer risk. *PLoS One* **12**, e0175535 (2017).
- L. Y. Hao et al., Short telomeres, even in the presence of telomerase, limit tissue renewal capacity. *Cell* **123**, 1121–1131 (2005).
- J. W. Shay, W. E. Wright, Hallmarks of telomeres in ageing research. *J. Pathol.* **211**, 114–123 (2007).
- N. Barker, A. van Oudenaarden, H. Clevers, Identifying the stem cell of the intestinal crypt: Strategies and pitfalls. *Cell Stem Cell* **11**, 452–460 (2012).
- I. L. Cameron, Cell proliferation and renewal in aging mice. *J. Gerontol.* **27**, 162–172 (1972).
- N. S. Wolf, P. E. Penn, D. Jiang, R. G. Fei, W. R. Pendergrass, Caloric restriction: Conservation of in vivo cellular replicative capacity accompanies life-span extension in mice. *Exp. Cell Res.* **217**, 317–323 (1995).