The Ink4a/Arf locus encodes 2 tumor suppressor molecules, p16^{INK4a} and Arf, which are principal mediators of cellular senescence. To study the links between senescence and aging in vivo, we examined Ink4a/Arf expression in rodent models of aging. We show that expression of p16^{INK4a} and Arf markedly increases in almost all rodent tissues with advancing age, while there is little or no change in the expression of other related cell cycle inhibitors. The increase in expression is restricted to well-defined compartments within each organ studied and occurs in both epithelial and stromal cells of diverse lineages. The age-associated increase in expression of p16^{INK4a} and Arf is attenuated in the kidney, ovary, and heart by caloric restriction, and this decrease correlates with diminished expression of an in vivo marker of senescence, as well as decreased pathology of those organs. Last, the age-related increase in Ink4a/Arf expression can be independently attributed to the expression of Ets-1, a known p16^{INK4a} transcriptional activator, as well as unknown Ink4a/Arf coregulatory molecules. These data suggest that expression of the Ink4a/Arf tumor suppressor locus is a robust biomarker, and possible effector, of mammalian aging.
In particular, mediators of senescence, an important tumor suppressors, are thought to play a role in human aging (reviewed in ref. 2). Tissue regeneration. Therefore, regulators of stem cell longevity homeostasis depends on the persistent and obligate function of soma, such as Drosophila and Caenorhabditis elegans, mammalian homeostasis depends on the persistent and obligate function of reservoirs of self-renewing tissue stem cells that are necessary for tissue regeneration. Therefore, regulators of stem cell longevity are thought to play a role in human aging (reviewed in ref. 2). In particular, mediators of senescence, an important tumor suppressor mechanism and specialized form of growth arrest, have been suggested to play a role in aging by limiting the long-term growth of these self-renewing compartments (reviewed in refs. 3, 4). For example, excess activity of p53 has been shown to induce premature aging in mice in multiple tissue types (5, 6). For these reasons, it has been argued that some aspects of aging may result from the beneficial, anticancer functions of mediators of senescence such as p53, ARF, and p16INK4a (7). The age-related increase in expression of p16INK4a and ARF is attenuated in the kidney, ovary, and heart by caloric restriction, and this decrease correlates with diminished expression of an in vivo marker of senescence, as well as decreased pathology of those organs. Last, the age-related increase in Ink4a/Arf expression can be independently attributed to the expression of Ets-1, a known p16INK4a transcriptional activator, as well as unknown Ink4a/Arf coregulatory molecules. These data suggest that expression of the Ink4a/Arf tumor suppressor locus is a robust biomarker, and possible effector, of mammalian aging.

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**Introduction**

Aging is a complex set of phenotypes characterized by reduced repair and/or regeneration of lost or damaged cells. Although studies in lower organisms have linked metabolism and the production of oxygen radicals with the rate of aging (reviewed in ref. 1), less is known about the molecular effectors of aging in mammals. As opposed to homeostasis in the molecular effectors of aging in mammals. As opposed to homeostasis in the molecular effector of aging in mammals, such as Drosophila and Caenorhabditis elegans, mammalian homeostasis depends on the persistent and obligate function of reservoirs of self-renewing tissue stem cells that are necessary for tissue regeneration. Therefore, regulators of stem cell longevity are thought to play a role in human aging (reviewed in ref. 2). In particular, mediators of senescence, an important tumor suppressor mechanism and specialized form of growth arrest, have been suggested to play a role in aging by limiting the long-term growth of these self-renewing compartments (reviewed in refs. 3, 4). For example, excess activity of p53 has been shown to induce premature aging in mice in multiple tissue types (5, 6). For these reasons, it has been argued that some aspects of aging may result from the beneficial, anticancer functions of mediators of senescence such as p53, ARF, and p16INK4a.

We sought to examine the role of p16INK4a, ARF, and related cell cycle inhibitors in mammalian aging. This choice was motivated by the importance of p16INK4a and/or ARF in the senescence in vitro of many murine and human cell types, e.g., islets (7), fibroblasts (8, 9), keratinocytes (10, 11), and macrophages (12); the fact that expression of p16INK4a significantly accumulates with normal aging in rodents and humans in a variety of tissues (13–19); and the fact that p16INK4a and Arf appear to be principal mediators of stem cell longevity in vivo (20–24). Toward this end, we sought to define the pattern of expression of cell cycle inhibitors in aging rodents, and to determine the effects of known regulators of aging such as caloric restriction on the expression of these inhibitors of proliferation.

**Results**

Expression of Ink4a/Arf in vivo. We determined the expression of cyclin-dependent kinase inhibitor (CDKI) family members, which are principal regulators of the mammalian cell cycle (25). CDKIs inhibit the kinase activity of Cdk2, Cdk4, and Cdk6, thereby inhibiting phosphorylation of their target protein Rb and eliciting G1 arrest. Eighteen quantitative real-time PCR strategies (Supplemental Table 1; supplemental material available at http://www.jci.org/cgi/content/full/114/9/1299/DC1) were used to assay CDKI expression in tissues from old and young syngeneic adult rodents (Figure 1, A and B). We also tested the expression of Arf, a cell cycle inhibitor through its regulation of p53 stability, because Arf is coregulated with p16INK4a (Figure 1, A and B), and its expression has previously been shown to increase with aging in the mouse (18).

A marked increase (3-fold or greater) in the expression of p16INK4a was seen in 26 of 27 organs analyzed from 15 murine and 12 rat tissues. Particularly large (>30-fold) increases in relative terms of the ratio of RNA expression in old versus young tissues (old/young ratio) were seen in the murine cecum, kidney, ovary, and uterus (Figure 1A; log2 scale), while the highest expression in absolute terms was seen in lung, lymph node, adrenal, and uterus from aged animals (Figure 1B; log10 scale). The geometric mean of the old/young ratios among the 15 murine tissues analyzed was 9.7 (i.e., the average tissue demonstrated an approximately 10-fold increase in the expression of p16INK4a with aging). This value is likely an underestimate of the true average fold increase, because in tissues such as the pancreas and bone marrow (Figure 1A), p16INK4a expression was below the level of detection in young animals. Therefore, in these tissues, only a minimum estimate of the fold increase in expression in these tissues could be determined. Similarly, Arf expression increased severalfold in most of the tissues examined, particularly heart, duodenum, kidney, and uterus (Figure 1, A and B). The geo-
The metric mean of the Arf old/young ratios was a 3.5-fold increase, while the next highest cell cycle inhibitor, p21CIP, demonstrated only a 1.4-fold average increase. These data do not exclude a specific role for another CDKI in a particular tissue; for example, p15INK4b showed an approximately 5-fold increase in expression in the heart with aging. Likewise, our data do not exclude the possibility that certain of the CDKIs (e.g., p18INK4c [ref. 26] or p27KIP [ref. 27]) are regulated predominantly in a posttranscriptional manner with aging. Nonetheless, Ink4a/Arf upregulation appears to be a strong correlate to organismal aging across many tissue types, and this marked and widespread upregulation is unique among the major in vivo inhibitors of the mammalian cell cycle.

In terms of absolute transcript number and protein expression, the expression of p16INK4a and Arf was considerably lower in tissues from aged mice than in primary cultures of murine embryo fibroblasts (MEFs) at early (P4) and late (P7) passage are shown for comparison. Maximum estimated expression is indicated, as expression was below the level of detection.

**Figure 1**

Expression of the Ink4a/Arf locus increases with aging. (A) Relative expression. The ratios (log₂ scale) of the expression of cell cycle inhibitors — old (26 months)/young (2.5 months) — from 15 tissues is graphed ± SEM. Each estimate represents the mean of 8–32 quantitative RT-PCR reactions on independent RNA samples derived from 4–6 mice. *Minimum estimate of old/young ratio. (B) Absolute expression. The absolute copy number of p16INK4a and Arf mRNA molecules (log₁₀ scale) per 90 ng total RNA RT-PCR from 15 tissues of young (2.5 months) and old (26 months) mice is graphed ± SEM. Murine embryo fibroblasts (MEFs) at early (P4) and late (P7) passage are shown for comparison. #Maximum estimated expression is indicated, as expression was below the level of detection.
expression increases only in a relatively small subset of cells within a given tissue (e.g., the β cells of the pancreas; Figure 2A and ref. 17). To determine in which organ compartments the expression of Ink4a/Arf increased, we performed additional lines of analysis including immunohistochemistry (IHC) and mRNA quantification in purified populations of sorted cells (Figure 2, A and B, and data not shown). Using these approaches, we were able to define the compartmental expression of p16INK4a and/or Arf in selected tissues from aging rodents (summarized in Table 1).

This analysis led to several conclusions. First, there was a good correlation in most tissues between mRNA expression and protein expression. An exception, however, was the lung, where RNA expression of p16INK4a even from young mice was higher in absolute terms than in several tissues from old mice (Figure 1B) yet protein expression was not detected by IHC or immunoprecipitation–Western analysis (not shown). This result could be attributable to either translational regulation of p16INK4a (29) or decreased protein stability in this tissue. Furthermore, we detected p16INK4a expression in a variety of cell types, including lining epithelium (e.g., of the renal cortex), mesenchyme (of the uterus), lymphocytes (in the spleen and lymph nodes), and specialized endocrine secretory tissues (e.g., pancreatic islet and adrenal) (Figure 2, A and B; Table 1). For example, in the uterus, there was marked expression of p16INK4a in the stroma of aged mice, but less so by IHC in the uterine epithelium. In the kidney, a marked increase in expression was noted in the cortical tubules, with detectable, but significantly less, expression in the medulla and glomeruli. In the spleen, increased expression of p16INK4a in both the stromal (red pulp) and lymphocyte (white pulp) compartments was noted, although this was higher in stroma (Figure 2, A and B). In aggregate, these data suggest that the expression of p16INK4a mRNA and protein increases in cells of varied histogenetic origins with aging. These results indicate that expression of the Ink4a/Arf locus can be induced in many if not most cell types in vivo, as would be suggested by related in vitro observations (7–12), as well as by the locus’s role in the suppression of a wide variety of cancer types (30).

To permit quantification of the compartmental expression of p16INK4a, hematopoietic organs were harvested and specific cell types purified (Figure 2B). Expression of p16INK4a increased with aging in both the stromal and the cellular (predominantly T cell) compartments of lymph nodes, but to a greater degree in the stroma (42-fold vs. 13-fold). Similarly, both the stromal and the cellular compartments of the spleen showed increased expression of p16INK4a with aging (15-fold vs. 5-fold). Among the splenic cellular elements, the predominant increases were seen in lymphocytes (B220+, 48% of total splenic cells) and cells that did not express B lymphocyte or myeloid markers (double negative for Mac1 and B220, 22% of cells), whereas little increase was seen in the myeloid compartment (Mac1+, 9% of cells). Lastly, we compared the expression of p16INK4a and Arf in lineage-negative (2%) versus lineage-positive (98%) bone marrow cells. In this compartment, the principal increase in expression was in lineage-negative cells, which are enriched for hematopoietic stem and progenitor cells (HSPCs) (31). This finding is consistent with the reported role of Ink4a/Arf expression in HSPCs and early committed progenitors from adult mice (21). Therefore, Ink4a/Arf can be expressed in several cell types of hematopoietic organs, including stromal elements, lymphocytes, and progenitor cells.
Caloric restriction retards p16INK4a accumulation. Caloric restriction (CR) retards aging in many species, but the molecular effectors of this are unknown (32). To examine the effects of CR on p16INK4a expression, we harvested total RNA from organs from caloricly restricted (CR) and ad libitum–fed (AL) Fischer 344 (F344) rats (Supplemental Figure 1, A and B) (33). We confirmed that Ink4a/Arf expression increased with aging in 12 different tissues from AL rats (representative data shown in Figure 3A). There was excellent concordance in the fold increase of Ink4a/Arf and p21CIP expression between mouse and rat tissues from AL animals, except in the testis. In murine testis, no increase in p16INK4a was seen (Figure 1, A and B), whereas in rat testis, a greater-than-135-fold increase in p16INK4a expression was noted (Figure 3A). A histologic examination of rat testis from old AL males, however, demonstrated marked involvement with Leydig cell tumor (Supplemental Figure 1C), which occurs spontaneously in the ever, demonstrated marked involvement with Leydig cell tumor (34), which happens in the mouse. Nonetheless, in accord with our observation, a chronic disease seen with nearly complete penetrance in aged F344 rats and a principal limit to the longevity of this strain (34). Therefore, in both of these tissues, the decrease in Ink4a/Arf expression occurred in concert with a CR-induced reduction of a disease state, suggesting that increased Ink4a/Arf expression in some circumstances may predispose to organ pathology, pre-saging organ failure and death. These observations also predict that tissue-specific increases in Ink4a/Arf expression with aging likely will vary among mammals (and even across inbred strains of rodents) to reflect specific predispositions to disease resulting from unshared genetics and/or environmental exposures.

We also tested the relationship of Ink4a/Arf expression with aging in the kidney and expression in the lung, lymph node, spleen, and liver from AL animals, except in the testis. In murine testis, no increase in p16INK4a was seen (Figure 1, A and B), whereas in rat testis, a greater-than-135-fold increase in p16INK4a expression was noted (Figure 3A). A histologic examination of rat testis from old AL males, however, demonstrated marked involvement with Leydig cell tumor (Supplemental Figure 1C), which occurs spontaneously in the mouse. Nonetheless, in accord with our observations in the rat, CR significantly reduced Ink4a/Arf expression in both lung and kidney from unshared genetics and/or environmental exposures.

When compared with tissues from AL animals, several, but not all, tissues from CR animals showed marked reductions in the age-induced increase of Ink4a/Arf expression. A 2- to 16-fold attenuation in the age-induced expression of p16INK4a was seen in adrenal, heart, kidney, ovary, and testis; but CR did not attenuate Ink4a/Arf expression in the lung, lymph node, spleen, and liver (Figure 3A). Unexpectedly, in the uterus, CR appeared to induce a significant increase in Ink4a/Arf expression with aging. Consistent with a previous report (34), we noted that aged CR males demonstrated greatly reduced Leydig cell hyperplasia (Supplemental Material Figure 1C) compared with AL animals. Correspondingly, this decreased tumor burden correlated with a marked reduction in the age-induced increase in p16INK4a expression (135-fold in AL rats vs. 58-fold in CR rats). Therefore, the decrease in p16INK4a expression with CR in aged rat testis likely results from an antineoplastic effect of CR. In contrast, the marked decrease in p16INK4a expression in the cortical tubules of the kidney (Figure 3B) from CR animals correlated with decreased nephritis (not shown), a chronic disease seen with nearly complete penetrance in aged F344 rats and a principal limit to the longevity of this strain (34). Therefore, both of these tissues, the decrease in Ink4a/Arf expression occurred in concert with a CR-induced reduction of a disease state, suggesting that increased Ink4a/Arf expression in some circumstances may predispose to organ pathology, pre-saging organ failure and death. These observations also predict that tissue-specific increases in Ink4a/Arf expression with aging likely will vary among mammals (and even across inbred strains of rodents) to reflect specific predispositions to disease resulting from unshared genetics and/or environmental exposures.

Furthermore, we sought to determine the functional significance of Ink4a/Arf expression in aging mammals. We used a well-

<table>
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<th>Table 1</th>
<th>Expression of p16INK4a in murine tissues (present work) as compared with published human IHC data</th>
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<td>Tissue</td>
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<td>Uterus</td>
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Abs. exp., absolute expression; ND, not determined; Duod., duodenum; Panc., pancreas; tr., trace. ACopies per 90 ng total RNA.

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Effects of caloric restriction and GHR deficiency on gene expression and aging. (A) Relative expression ratios (old/young, log₂ scale) of cell cycle inhibitors in 7 tissues derived from old (28 months) and young (3 months) AL or CR F344 rats. The relative ratios are graphed ± SEM. Each estimate represents the mean of 8–16 quantitative RT-PCR reactions on independent RNA samples derived from 4 rats. (B) Immunoperoxidase staining on paraffin-embedded kidney sections from young, old AL, and old CR F344 rats using an anti-p16INK4a antibody. G, glomeruli seen in cortical sections. (C) SA-β-gal staining in AL and CR mouse and rat kidney. C, renal cortex; M, renal medulla. Thin tissue slices were stained for mice, as opposed to small tissue wedges for rats. SA-β-gal activity is predominantly restricted to the renal cortex. (D) Relative expression ratios (old/young, log₂ scale) of Ets-1 in kidneys derived from AL and CR rats and mice. Results from the kidneys from AL and CR mice with and without GHR deficiency are also shown. Each estimate represents the mean of 8–32 quantitative RT-PCR reactions on independent RNA samples derived from 8 mice or 4 rats.

The expression of Ink4a/Arf regulators was next considered (Supplemental Figure 3). A weak negative correlation was found between the expression of Id1 and that of p16INK4a (r = −0.40, P = 0.07), consistent with Id1’s known role as a transcriptional repressor of p16INK4a (40, 42). No significant correlation was seen between Bmi-1 expression and that of either p16INK4a or Arf (not shown). Remarkably, however, a strong correlation was noted between p16INK4a and Ets-1 (r = 0.62, P < 0.001; Figure 4A), but not between Arf and Ets-1 (r = 0.15, P = 0.40). This observation is consistent with the finding that Ets-1 induces the expression of p16INK4a, but not Arf, in cultured cells (40, 41), and that of p21CIP1 was limited in most tissues (e.g., liver and spleen) because of high background activity but was interpretable in both murine and rat tissues (18), we are not aware of previous data showing coordinated expression in vivo. These results are consistent with the known transcriptional relationships of these genes; in particular, several proteins (43–47) have been identified that coordinate the expression of both Ink4a/Arf products, and Arf regulates p53 activity, which in turn induces p21CIP expression. Unexpectedly, we found a strong correlation between p16INK4a and p21CIP (r = 0.68, P = 0.002), which suggests that these genes are regulated by common or related transcriptional elements. Although these homologous INK4s are concomitantly expressed in many tissues (18), we are not aware of previous data showing coordinated expression in vivo.

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a p16INK4a-specific effect that correlates with Ets-1 expression, and an independent, Ink4a/Arf coregulatory effect.

Discussion
This work adds to a growing body of evidence suggesting that in vivo senescence, induced by Ink4a/Arf expression, plays a causal role in the aging of certain tissue types. First, as shown in this work, Ink4a/Arf expression is not only tightly linked with aging but is influenced by CR and correlates with SA-β-gal expression. Ink4a/Arf expression has also been shown to increase in several tissues in a murine model of premature aging (48), and expression of p16INK4a or Ink4a/Arf correlates in vivo with impaired proliferation and failure of hematopoietic stem cells (20, 21, 23, 24). While the functional consequences of Ink4a/Arf expression in aging tissues are an area of active investigation, p16INK4a deficiency has been shown to ameliorate an age-related decline in T cell responsiveness to CD3 and CD28 (49), a hallmark of aging in the immune system (50). Similarly, neural stem cells from Bmi-1-deficient animals demonstrate increased Ink4a/Arf expression and impaired regenerative potential; and this phenotype can be partially rescued by p16INK4a deficiency (22). In aggregate, these results suggest that p16INK4a and/or Arf expression is not merely a biomarker, but also an effector, of aging, presumably by limiting the self-renewal capacity of disparate tissues including at least lymphoid organs, bone marrow, and brain. Further determination of the functional role of the Ink4a/Arf locus in other aging tissues, as well as the relative contribution p16INK4a versus Arf, will require further study of mice with germ-line and tissue-specific deletion of p16INK4a and/or Arf.

We detected a significant attenuation of age-induced Ink4a/Arf expression by CR in several tissues, but particularly in the kidney, where the effect was nearly complete. The reduction in Ink4a/Arf expression correlated with decreased nephritis in aged F344 rats, a major limit of longevity in this strain (34). Therefore, it is tempting to speculate that Ink4a/Arf expression limits the ability of aged kidney to self-repair, resulting in organ failure and death. Our analysis of tissue from GHR-deficient animals was too limited to determine the effects, at the organismal level, of GHR deficiency on Ink4a/Arf expression; but in contrast to CR, GHR loss was not seen to affect p16INK4a expression in the aging kidney (Supplemental Figure 2). It is possible that reductions in IGF-1 similarly retard the increase in Ink4a/Arf expression in other tissues; this would explain the ability of CR to extend the longevity of IGF-1–deficient animals (36). Alternatively, IGF-1 deficiency may work through a distinct, Ink4a/Arf-independent mechanism to retard aging.

As rodents have large telomeres and promiscuously express telomerase (51, 52), the signal that induces Ink4a/Arf-mediated senescence in the kidney and other organs is not likely to be telomere-based. We were, however, able to demonstrate a strong correlation between Ets-1 expression and p16INK4a expression with aging across 27 different tissues from 2 species, with and without CR. Given that Ets-1 is a direct transcriptional activator of p16INK4a in vitro (40, 41), these data suggest that Ets-1 is a principal determinant of p16INK4a expression with aging in vivo. Ets-1, in turn, is regulated by a variety of stress-related signals and, in particular, MAPK activity. Ets-1 activation has been linked to both ERK (53, 54) and p38MAPK (55) signaling, while recent genetic evidence has shown that p38MAPK activation is associated with increased Ink4a/Arf expression in vivo (56). These observations are consistent with the model (Figure 4B) that presently unidentified stresses activate Ets-1 via MAPK pathways to induce a p16INK4a-mediated senescence in aging organisms.

By statistical methods, we additionally inferred the existence of an unknown coregulatory factor(s) that is distinct from Ets-1 and that modulates the expression of both p16INK4a and Arf with aging. An obvious candidate is the Polycomb group (PcG) protein Bmi-1, which has been shown to regulate p16INK4a and Arf expression in stem cells in vivo (20–22, 57). We did not, however, detect a negative correlation between Bmi-1 expression and either p16INK4a or Arf in most aging tissues. Therefore, transcription of Bmi-1 is not likely to be a predominant regulatory mechanism of Ink4a/Arf expression in vivo across multiple tissue types, but this analysis does not exclude a specific role for Bmi-1 in a particular tissue. Along these lines, we detected greater-than-2-fold decreases in Bmi-1 expression with aging in spleen and bone marrow (Supplemental Figure 3), which suggests that perhaps Bmi-1–containing PcG complexes are of particular importance in repressing Ink4a/Arf expression in hematopoietic tissues, as has been suggested (20, 21, 43). Likewise, it is possible that related
Ink4a/Arf-repressing PcG family members such as Cbx7 (58) or Mel18 (59) subsume the function of Bmi-1 in other tissues; that PcG complex activity is regulated post-transcriptionally; or that unidentified non-PcG molecules are important coregulators of Ink4a/Arf. In this regard, it will also be of future interest to determine the links, if any, between Ink4a/Arf expression and known regulators of aging such as Foxo proteins and SirT1 (60, 61). A molecular biomarker of physiologic, as opposed to chronologic, age is needed in clinical medicine. While other molecular markers of aging have been suggested, we believe the analysis of Ink4a/Arf expression may prove particularly fruitful in this regard, for several reasons. First, the change in p16INK4a expression with aging is large—over 10-fold in many tissues— and relatively simple to measure by either immunohistochemical methods or quantitative real-time PCR analysis. For example, there is a clinically validated, commercially available kit (DakoCytomation Inc.) for use in the detection of p16INK4a. Many anatomic pathologists are already comfortable with the interpretation of p16INK4a IHC. Moreover, our results demonstrate that Ink4a/Arf can be expressed in many cell types in response to age-induced stresses, and that Ink4a/Arf expression changes in at least a subcompartment of the majority of mammalian organs with aging. For this reason, p16INK4a or Arf expression may be a particularly robust marker of aging, useful for the study of many cell types in disparate organs. Lastly, given the probable causal role of p16INK4a and/or Arf in aging, expression of Ink4a/Arf should be a stronger correlate of aging than expression of other genes whose expression is merely epiphenomenal.

One anticipates that a well-defined molecular marker of aging could be used for at least 4 clinical purposes: (a) to facilitate the forecasting of disease progression in premorbid syndromes such as renal insufficiency and cardiomyopathy; (b) to provide a surrogate marker for efficacy of anti-aging therapeutics; (c) to predict future toxicity from noxious therapies such as chemo- or radiotherapy and surgery that require tissue regeneration and repair; and (d) to determine donor suitability for bone marrow, solid organ, and tissue allografts. The utility of Ink4a/Arf expression in predicting, and perhaps determining, a tissue’s future regenerative potential—i.e., indications (c) and (d) above—is a topic of active, ongoing study. This work, however, does serve as “proof of principle” for the use of Ink4a/Arf expression for indications (a) and (b): we demonstrate that the expression of Ink4a/Arf correlates with tissue aging and alterations in disease progression in the rodent kidney and testis, and that reduced expression of the locus correlates with response to an anti-aging therapy, caloric restriction. Therefore, our data suggest that the measurement of Ink4a/Arf expression may similarly be of clinical benefit in the determination of human physiologic age.

Methods

Animals. All animals were housed and treated in accordance with protocols approved by the institutional care and use committee for animal research at the University of North Carolina. All murine analyses were performed in C57BL/6 mice unless otherwise mentioned. For cell sorting experiments, 22- to 25-month-old mice of mixed genetic background (C57BL/6 x NIH Black Swiss; from Yue Xiong, University of North Carolina) were analyzed. Old (21 months) and young (5 months) GHR KO and WT (CR and AL) mice of mixed genetic background (derived from Ola-BALB/c, C57BL/6, and C3H; courtesy of John Kopchick, Ohio University, Athens, Ohio, USA) were analyzed. CR for this murine cohort was done as previously described (38). Aged rodents were obtained from the aged-rodent colonies of the National Institute on Aging (ref. 33; see also http://www.nia.nih.gov/research/rodent.htm) and euthanized at our facility, and organs were harvested as described below. For murine studies, old (25–26 months) and young (2.5–3.5 months) C57BL/6 mice (3 males and 3 females) were analyzed. For CR experiments, old (28 months) and young (3 months) F344 AL rats (2 old and 2 young males, 2 old and 2 young females) were obtained, as well as old (28 months) CR littermates (2 males and 2 females).

Animals were euthanized by CO2 inhalation, and organs were harvested. Organs were grossly dissected free of contaminating tissue (e.g., adipose) and then snap-frozen. RNA was harvested from a matched portion of the organ (e.g., the superior half of the kidney) in all instances to assure that results did not differ because of organ composition. For RNA analysis, tissues were collected after cardiac perfusion with RNALater (Ambion Inc.) or after snap-freezing in liquid nitrogen. For IHC and H&E staining, organs were harvested and fixed as described below, and then stored in 70% ethanol until paraffin embedding.

Quantitative real-time PCR. Total RNA was extracted from tissues, serially passaged murine embryo fibroblasts (each passage corresponds to 3 days in vitro), or sorted cell samples using a QIAGEN RNaseq RNA isolation kit according to the manufacturer’s instructions. Transcription into cDNA was done in a 20-μl volume using oligo-dT12-18 or random hexamer and ImProm-II reverse transcriptase (Promega Corp.) according to the manufacturer’s instructions. All PCR reactions were carried out in a final volume of 20 μ1 and were performed in duplicate for each cDNA sample in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) according to the manufacturer’s protocol. All experiments were done on organs from at least 2 different animals in each group (old vs. young, CR vs. AL).

Sequence-specific primers and probe were designed using Primer Express (Applied Biosystems) for the indicated genes (Supplemental Table 1). Oligonucleotide primers and probes were synthesized by MWG Biotech. All primer sets were designed to span an intron. Predeveloped assays were purchased from Applied Biosystems for the additionally listed murine and rat genes (Supplemental Table 1). The reaction mix consisted of Universal Master Mix No AmpErase UNG (Applied Biosystems), 0.25 μM fluorescent probe, 0.9 μM of each specific forward and reverse primer, and 9 μl of diluted cDNA (equivalent to ~90 ng total RNA). Amplifications were done under standard conditions. The number of PCR cycles needed to reach the fluorescence threshold was determined in duplicate for each cDNA, averaged, and then normalized to at least 1 reference gene (18S, GAPDH, or TATA-binding protein [TBP]; see Supplemental Figure 4 for further details) to yield the cycle number at which fluorescence threshold was reached (C). The ratio of expression in old versus young tissues (old/young) was determined as 2 (C(old) – C(young)), therefore, log2(oldd/young) = C(old) – C(young). Ratios were log-transformed for calculations of SE and Pearson correlation coefficients using Prism software (GraphPad Software Inc.). For absolute quantification of transcript copy number for p16INK4a, p15INK4b, p19ARF, p21CIP1, p18INK4c, and p19INK4d, the fragment of interest was cloned and a standard curve generated with serial 4-fold dilutions (Supplemental Figure 5). For all assays tested, the PCR reaction was linear over the range studied (19–40 cycles of amplification; Supplemental Figure 5). If C was not reached by 40 cycles, expression was considered below the limit of detection. All RT-PCR reactions gave a single band when analyzed by gel electrophoresis, and all reactions used a fluorochrome-labeled internal probe to enhance sensitivity and specificity.

Cell sorting of bone marrow, lymph node, thymus, and spleen. Tissues from 5- and 22-month-old mice (n = 2 per age) were disaggregated and sorted. For bone marrow, lineage-negative and lineage-positive fractions were collected using a lineage cell depletion kit with an autoMACS Separator (Miltenyi Biotec Inc.) according to the manufacturer’s protocol. Stromal and cell components for lymph node, thymus, and spleen were prepared by pressing between frosted glass slides, followed by washing with PBS containing 10% FCS. Splenocytes...
were then further analyzed after red blood cell lysis by staining with anti-B220 (Caltag Laboratories Inc.) and anti-CD11b (Mac-1; Caltag Laboratories Inc.) mAbs for 30 minutes on ice followed by washing. Cells were sorted by MoFlo (Cytomation Inc.) to B220+, Mac-1+, and B220 Mac-1 populations (purity >94%) at the University of North Carolina FACS Core facility.

IHC and SAβ-gal staining. To determine optimal IHC staining conditions for p16INK4a, tissues were harvested from age-matched littermate β-gal staining was performed as previously described (15, 39, 43). In brief, 4.3% glacial acetic acid, and 3.5% formalin) was determined to be optimal as previously described (62), Fekete’s acid alcohol solution (61% ETOH, to 0.5% glutaraldehyde/PBS (pH 6.0). After fixation, organ slices were washed twice in PBS (pH 6.0), stained overnight at 37°C in SAβ-gal buffer (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mM MgCl2 in PBS, pH 6.0), and photographed with a high-resolution CCD camera (LighTools Research).

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