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# Changes in gene expression associated with aging commonly originate during juvenile growth

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ABSTRACT

In mammals, proliferation is rapid in many tissues during early postnatal life, causing rapid somatic growth. This robust proliferation is then suppressed as the animal approaches adult size, bringing many tissues to a quiescent state where proliferation occurs only as needed to replace dying cells. Recent evidence suggests that the mechanism responsible for this decline in proliferation involves a multi-organ genetic program. We hypothesized that this genetic program continues to progress into later adult life, eventually suppressing proliferation to levels below those needed for tissue renewal, thus contributing to aging. We therefore used expression microarray to compare the temporal changes in gene expression that occur in adult mouse organs during aging to those occurring as juvenile proliferation slows. We found that many of the changes in gene expression that occur during the aging process originate during the period of juvenile growth deceleration. Bioinformatic analyses of the genes that show persistent decline in expression throughout postnatal life indicated that cell-cycle-related genes are strongly over-represented. Thus, the findings support the hypothesis that the genetic program that slows juvenile growth to limit body size persists into adulthood and thus may eventually hamper tissue maintenance and repair, contributing to the aging process.



## 1. Introduction

Aging is generally regarded as a gradual decline in physiological function throughout the organism, decreasing the ability to respond to stress and homeostatic imbalance, and increasing susceptibility to disease. Despite its obvious relevance to human health, the biological basis of aging is not well understood. At the organ level, aging is thought to be associated with a progressive decline in cell proliferation (Burton, 2009), one example being the decreasing capability of the aged liver to regenerate after partial hepatectomy (Stocker and Heine, 1971; Timchenko, 2009). This loss of renewal and regenerative potential may be in part due to decreased proliferative capacity of adult stem cells (Yui et al., 1998; Krtolica, 2005; Sharpless and DePinho, 2007) and to the accumulation of senescent cells with age (Jeyapalan et al., 2007).

Declining proliferative capacity in many tissues does not begin in mid or late adult life, as the aging process develops, but instead initiates much earlier (Chang et al., 2008). In embryonic and early postnatal life, many tissues show high replication rates, leading to rapid somatic growth of these organs. If this rate of proliferation were to remain constant, somatic growth would be exponential. Therefore, cellular mechanisms are required to slow this robust proliferation, such that, by adulthood, most tissues have entered a quiescent state where proliferation occurs only as needed to replace dying cells (Pellettieri and Sanchez, 2007). Recent evidence suggests that the mechanism responsible for this decline in juvenile cell proliferation involves a genetic program that is common to multiple organs (Lui et al., 2008, 2010; Finkielstain et al., 2009) and includes the downregulation of multiple growth-promoting genes with age. This genetic program appears to be driven by growth itself, since growth-inhibiting conditions delay the program (Finkielstain et al., 2009; Lui et al., 2010). Thus proliferation appears to cause downregulation of growth-promoting genes, which in turn limits proliferation.

The developmental theory of aging proposes that aging could be caused in part by continuing actions of developmental processes, which eventually produce adverse effects in postreproductive life (de Magalhaes and Church, 2005). In particular, there is evidence suggesting that cell proliferation beginning in early life may lead to damage or changes which in turn lead to a gradual loss of renewal capacity, contributing to aging (de Magalhaes and Faragher, 2008).

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Here, we considered the specific possibility that the mechanisms that progressively restrain juvenile growth may continue to progress into adult life, thus contributing to the decline in proliferative capacity associated with aging. Progression of the underlying proliferation-limiting genetic program might lead first to cellular quiescence in young adulthood, such that some cells can still proliferate when stimulated for tissue renewal, but then, during aging, continued progression of the program might further limit the ability of cells to proliferate to a level below that is needed for tissue renewal. This hypothesis, which links juvenile growth and aging, might provide an explanation for the observation that small mammals generally undergo both aging and suppression of juvenile growth on a far shorter time scale than do large mammals. Similarly, the hypothesis might help explain why conditions that inhibit proliferation such as caloric restriction, growth hormone deficiency, or insulin-like growth factor-I deficiency, might delay the genetic program and thus conserve proliferative capacity and slow aging (Mote et al., 1991; Brown-Borg et al., 1996; Coschigano et al., 2000; Flurkey et al., 2001; Smith et al., 2004; Spindler and Dhahbi, 2007). Finally this hypothesis is consistent with the antagonistic pleiotropy theory, which proposes that aging results from genes that have beneficial effects in early life, when natural selection is strong, but harmful effects at a later age when selection pressure is weaker (Williams, 1957).

As an initial test of this hypothesis, we used expression microarray to analyze changes in gene expression that occur in liver, kidney and lung of mice during aging. We then compared these changes to those occurring in early postnatal life, as somatic growth decelerates. We found that many of the changes in gene expression that occur during aging originate in early postnatal life, during the juvenile period of growth deceleration. Furthermore, bioinformatic analysis suggested that a subset of genes that show consistent changes in expression in multiple juvenile organs and also in aging organs regulate cell proliferation. Thus, the findings support the hypothesis that the genetic program that slows growth in juvenile life in order to limit adult body size persists into adulthood, and may eventually hamper maintenance and repair of multiple organs.

## 2. Materials and methods

### 2.1. Animal procedures and tissue processing

C57BL/6 male mice at 1, 3, and 9 months of age were obtained from Charles River Laboratory (Charles River Laboratory, Wilmington, MA) and provided with regular chow (mouse chow 5R31, Purina LabDiet) and water *ad libitum*. All animals were maintained and used in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2003). Mice were killed by carbon dioxide inhalation at 3, 9, and 15 months of age. Liver, kidney, and lung were excised, homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) and stored at −80 °C for later use.

### 2.2. RNA extraction and purification

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions followed by RNeasy Mini Kit purification (QIAGEN, Valencia, CA) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometry at 260 nm. All RNA samples had a 260/280 nm ratio between 1.9 and 2.1. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and only high quality RNA (28S/18S ratio >1.8) was used for further analysis.

### 2.3. Microarray analyses

RNA was processed and analyzed by the NIDDK Core Facility at the National Institutes of Health using Affymetrix Mouse Genome 430 2.0 Array GeneChips (45,000 probe sets, Affymetrix, Santa Clara, CA). Three chips were used for each organ at each time point. Each microarray chip was used to analyze a pooled sample of RNA (2 μg) derived from three animals. Therefore, a total of nine animals were used. This pooling served to decrease biological variability. Microarray signals were analyzed using the Affymetrix RMA algorithm. ANOVA was performed and FDR reports were generated using Partek Pro software (Partek, St. Charles, MO). The FDR was calculated collectively using data from all three organs. Pathway analyses were done using Ingenuity Pathways Analysis Software 7.1 (Ingenuity Systems Inc., Redwood City, CA). Correlation analyses (Pearson's pairwise comparison and Spearman's rank correlation) and heat maps were generated using JMP 8 software (SAS Institute Inc., Cary, NC).

### 2.4. Quantitative real-time RT-PCR

Real-time PCR was used to assess specific mRNA levels in murine organs. Liver, kidney, and lung were dissected from C57BL/6 mice at 3, 9, and 15 months of age ($n$ = 8–10 animals per time point). Total RNA (100–200 ng) was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting cDNA solution was diluted 10-fold and stored at −20 °C for later use. Quantitative real-time PCR was performed for *18S*, *Ccl5*, *Cxcl9*, *Peg3*, and *Ube2c* using the following assays employing specific FAM or VIC-labeled (18S rRNA) TaqMan probes (Applied Biosystems, Foster City, CA): *Ccl5*, Mm01302427_m1; *Cxcl9*, Mm00434946_m1; *Peg3*, Mm01337379_m1; *Ube2c*, Mm00835439_g1; and 18S rRNA, 4319413E. Reactions were performed in triplicate on cDNA derived from individual animals, using the ABI Prism 7900 sequence detection system instrument (Applied Biosystems) according to the manufacturer's instructions. The relative quantity of each mRNA was calculated using the formula: relative expression = $2^{-\Delta CT} \times 10^6$, where CT represents the threshold cycle and $\Delta CT$ = (CT of gene of interest) − (CT of 18S rRNA). Values were multiplied by $10^6$ for convenience of comparison. Data are presented as mean ± SEM. The effect of age on relative expression was assessed by ANOVA.

## 3. Results

In this study, we analyzed the gene expression profile of aging C57BL/6 mice in liver, kidney and lung, representing organs with widely differing physiological functions and embryonic derivations. Time points were chosen to represent early (3-month-old), middle (9-month-old) and late (15-month-old) adulthood. The survival rate at 15 months of age was still 100% without apparent illness. We avoided time points close to the maximum life expectancy of these mice to reduce the possibility of picking up changes in gene expression associated with specific pathologic conditions rather than normal physiological aging.

### 3.1. Gene profiling of aging organs

Changes in gene expression during aging were analyzed using Affymetrix expression microarray that assesses 39,000 transcripts representing 34,000 genes in the mouse genome. We first identified genes that showed significant changes with age using a cutoff of $P < 0.0077$, which gave a false discovery rate of <10%. An additional cutoff of fold change ≥1.5 was used, so that the genes included are more likely to have important physiological effects. In each organ, hundreds of genes changed significantly during aging, with more genes being upregulated with age than downregulated (Table 1). Using Ingenuity Pathway Analysis (IPA), we identified the biological pathways and molecular functions that were over-represented by these age-related genes. This analysis implicated more than 30 high-level biological functions that were being altered significantly with aging of each organ, many of which are disease related (Fig. 1). The levels of expression in aging organs and the fold-changes with age were generally consistent with similar prior microarray studies (Supplemental Table 1) (Misra et al., 2007; Schumacher et al., 2008).

In general, each organ showed a distinct pattern of age-related biological functions. For example, lipid metabolism was strongly

**Table 1**
Number of genes that showed significant change in expression ($P < 0.0077$, FDR < 10%, ≥1.5-fold) with age (3–15 month) in each organ.

| Organ | Upregulated | Downregulated | Total |
|---|---|---|---|
| Liver | 381 (61%) | 247 (39%) | 628 |
| Kidney | 184 (71%) | 74 (29%) | 258 |
| Lung | 337 (64%) | 190 (34%) | 527 |