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Telomeres in Aging: Birds

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This chapter describes the use of avian species (the domestic chicken Gallus domesticus in particular) as model organisms for research in telomere biology and aging. Presented here are key concepts of avian telomere biology including characteristics of the model: the karyotype, telomere arrays, telomere shortening as a measure of the senescence phenotype or organismal aging, and telomerase activity in avian systems, including chicken embryonic stem cells, chicken embryo fibroblasts, the gastrula embryo and DT40 cells. Key methods used to measure telomere shortening and telomerase activity, and to conduct expression profiling of selected genes involved in telomere length maintenance are noted, as are methods for conducting gain- and loss-of-function studies in the chicken embryo. Tables containing references on general topics related to avian telomere biology and poultry husbandry as well as specific information regarding chicken orthologs of genes implicated in telomere maintenance pathways are provided. Internet resources for investigators of avian telomere biology are listed.

Introduction: The Chicken as a Model Organism

The versatility and utility of the domestic chicken as a developmental model was recently celebrated in a special issue of the journal *Developmental Dynamics* [(2004) 229, 413–712]. The chicken is one of the primary models for vertebrate developmental biology and a model organism for the study of virology, immunology, cancer and gene regulation (Tickle, 2004; Antin and Konieczka, 2005). With a 6.6X draft sequence of its genome completed, the chicken is poised to become even more valuable in traditional fields of study and also in aging research.

The earliest recorded descriptions of the chicken as a model for biological processes are attributed to Hippocrates and Aristotle, who wrote about embryonic development in fertilized chicken eggs. Twentieth-century embryologists authored numerous treatises describing, diagramming, and providing detailed photographs of the chicken during development (Hamburger and Hamilton, 1951; Romanoff, 1960; Eyal-Giladi and Kochev, 1976), which promoted use of the chicken embryo as a model for study of mechanisms including morphogenesis; neurogenesis; somatogenesis; limb, limb-digit and craniofacial development; left–right symmetry; axis development and others. The extensive use of the chicken as a model for early vertebrate development and its role in biomedical research has of necessity produced a detailed and comprehensive body of knowledge about basic chicken biology (Scanes *et al*., 2004; Stern, 2005). Add to all of this the accessibility of the chicken embryo, the relative economy of breeding and maintaining chickens and the ease of manipulation of embryonic and adult tissues and the chicken becomes an obvious choice as a model for the study of organismal and cellular senescence.

Aging and Replicative Senescence

Cellular or replicative senescence (*in vitro*) is often utilized as a model for the aging process (*in vivo*) due to the hypothesis that cellular aging recapitulates organismal aging (Wadhwa *et al*., 2005). The central dogma of replicative senescence holds that cultures of vertebrate fibroblasts have a limited capacity for proliferation. After a finite number of cell divisions, proliferation slows and culture arrest ensues. The barrier represented by culture arrest, termed the Hayflick Limit, is accompanied by a number of morphological changes including increased cell size, increased nuclear and nucleolar sizes, increased vacuolation of the cytoplasm and endoplasmic reticulum, expression of senescence-associated markers such as beta-galactosidase, and other changes in morphology and gene expression (Cristafalo *et al*., 2004 and references therein).

A genomic alteration associated with cellular or replicative senescence in a variety of organisms, including the chicken, is the shortening of telomeres (Prowse and Greider, 1995; Taylor and Delany, 2000; Swanberg and Delany, 2003). Shortened telomeres induce a DNA damage response, signaling cell cycle arrest. If the damage cannot be repaired, a checkpoint response results in further arrest or apoptosis. An alternative or complementary model for telomere-induced replicative senescence is loss of the protective effect of accessory proteins, such as TRF2, at the telomeres (Karlseder *et al*., 2002). Reactivation of telomerase or induction of the ALT (alternate lengthening of telomeres) pathway may provide protection against apoptosis or senescence and

facilitate transformation and immortalization by stabilizing telomeres (Swanberg and Delany, 2003 and references therein).

The prevailing explanation for telomere shortening, the end-replication problem, is based on the inability of DNA polymerase to replicate the ends of a linear chromosome, resulting in the incomplete replication of the 5′ end of the daughter strand. Telomerase is able to offset telomere shortening by adding telomere repeats to the parent strand which generates a longer telomere in the daughter strand. The telomerase holoenzyme is composed of two elements, telomerase rNA, TR, which contains the template for addition of telomeric repeats (Greider and Blackburn, 1989) and telomerase reverse transcriptase, TERT, the component which catalyzes the addition of repeats to the parent-strand chromosome end (Lingner *et al*., 1997). Most normal, adult vertebrate somatic cells, with the exception of cells from the lab mouse (*Mus musculus*), do not exhibit telomerase activity (Levy *et al*., 1992; Kim *et al*., 1994; Wright and Shay 2002; Levy *et al*., 1992). Not only does telomerase maintain telomeres of proliferating cells, it is also implicated in oncogenesis (Greider and Blackburn, 1989).

In addition to the end-replication problem and the compensating function of telomerase, telomere length is impacted by proteins that bind to and contribute to the architecture of the telomere. The thousands of duplex DNA telomere repeats are, for the most part, packaged in closely-spaced nucleosomes (Blackburn, 2001). However, the G-rich 3′ overhang assumes a terminal loop (t-loop), which displaces one of the duplex strands forming a related structure (D-loop). The D-loop t-loop is stabilized by telomere-binding proteins and their interaction partners (Greider 1999; Griffith *et al*., 1999; Wei and Price, 2003). Closed chromatin loops resembling t-loops have been observed in chicken using electron microscopy (Nikitina and Woodcock, 2004).

Telomere-repeat-binding factors 1 and 2 (TRF1 and 2) bind to double-stranded telomeric DNA (Wei and Price, 2003). TRF1, which induces telomeric DNA strands to bend, loop and pair (Bianchi *et al*., 1997; Smogorzewska *et al*., 2000), may produce shortening of telomeres by sequestering the 3′ overhang from telomerase (van Steensel and de Lange, 1997). TRF2 is described as protective of telomeres in some studies (Karlseder, 2003) and as a negative regulator of telomere length in other studies (Smogorzewska *et al*., 2000; Stansel *et al*., 2001). Overexpression of TRF1 or TRF2 produces a progressive shortening of telomeres (Ohki and Ishikawa, 2004 and references therein). Tankyrase 1 and 2 have the ability to bind TRF1, resulting in the ADP-ribosylation of TRF1 and the release of TRF1 from telomeric DNA. Overexpression of tankyrase 1 results in the removal of TRF1 from the telomeres followed by telomere elongation (Smith and de Lange, 2000).

In addition to the tankyrases, TRF1 and TRF2, Rap 1 and Pot 1 are involved in telomere maintenance. Rap1 interacts with TRF2, and Pot 1 may coat and protect both G-strand overhangs and the displaced G strand of a t loop (Bauman and Cech, 2001; Tan *et al*., 2003). Other proteins known to be relevant to telomere length regulation include c-myc, an oncogenic transcription factor which regulates cell proliferation, differentiation and apoptosis (Piedra *et al*., 2002). Down-regulation of c-myc is believe to be a prerequisite to differentiation (Skerka *et al*., 1993; Baker *et al*., 1994) and c-myc reactivates telomerase in transformed cells by inducing expression of its catalytic subunit TERT (Wu *et al*., 1999).

Chicken orthologs of TRF1 and 2, tankyrase 1 and 2, TR, TERT, c-myc, Rap 1 and Pot 1 have been characterized. In addition, chicken orthologs of the helicases that are missing or mutated in the progeroid disorders, Werner and Bloom Syndrome, have been identified but not studied. The Werner (WNR) and Bloom (BLM) proteins, both Req-Q helicases, have been implicated in telomere maintenance pathways (Du *et al*., 2004). Table 29.1 lists chicken genes related to telomere length regulation, their human orthologs and relevant references.

The Chicken as a Paradigm for Aging Research

Organisms frequently used in aging studies include yeast, *Drosophila*, *C. elegans*, and *M. musculus*, the laboratory mouse. With all of these well-characterized models available, particularly a mammalian vertebrate as well-studied as the lab mouse, why use an avian model? The advantages of using a vertebrate are obvious, and the mouse would at first glance appear to be a better choice than the chicken except for shortcomings of the mouse vis-à-vis the study of aging and oncogenesis. For example, mice have a very short lifespan. In contrast, maximum life expectancies of many species of birds approach the human life expectancy (Forsyth *et al*., 2002; Austad, 1997). Lifespan is significant, as cellular and genetic mechanisms governing cell proliferation are likely conserved in longer-lived species.

In addition to the issue of lifespan, laboratory mouse somatic cells retain telomerase activity and do not appear to display division-dependent telomere shortening (Prowse and Greider, 1995; Forsyth *et al*., 2002; Kim *et al*., 2002). Mouse models of telomere shortening have been developed, but it takes several generations in the telomerase knockout mouse (TR-/TR-) to achieve a phenotype that demonstrates division-dependent telomere shortening (Cheong *et al*., 2003). In contrast, human and chicken somatic cells lack telomerase, with down-regulation of telomerase occurring early in development. Division-dependent telomere shortening is established in chicken chromosomes (*in vivo* and *in vitro*) and human chromosomes. In human, mouse and chicken, highly proliferative tissues such as embryonic cells and intestine as well as transformed cells exhibit telomerase activity

TABLE 29.1
Chicken and human orthologs of genes involved in telomere maintenance pathways

Gene	Description	Accession	Chromosome or locus	References
hTERT	H. sapiens telomerase reverse transcriptase	AF015950	5p15.33	Nakamura *et al.*, 1007
cTERT	G. gallus telomerase reverse transcriptase	AY502592	2q21	Delany and Dainels, 2004
hTR	H. sapiens telomerase RNA	NR_001566	3q26	Feng *et al.*, 1995
cTR	G. gallus telomerase RNA	AY312571	9q-terminal	Delany and Daniels, 2003
tankyrase 1	H. sapiens tankyrase 1	NM_003747	8p23.1	Broccoli *et al.*, 1997
tankyrase 1	G. gallus tankyrase 1	AY142108	4	DeRyker *et al.*, 2003
tankyrase 2	H. sapiens tankyrase 2	AF438201	10q23.3	Kaminker *et al.*, 2001
tankyrase 2	G. gallus tankyrase 2	AY142107	unknown	DeRyker *et al.*, 2003
TERF1/TRF1	H. sapiens telomeric repeat binding factor 1	NM_003218	8q13	Zhong *et al.*, 1992
TERF1/TRF1	G. gallus telomeric repeat binding factor 1	AY237359	2	DeRyker *et al.*, 2003
TERF2/TRF2	H. sapiens telomeric repeat binding factor 2	BC024890	16q22.1	Broccoli *et al.*, 1997
TERF2/TRF2	G. gallus telomeric repeat binding factor 2	AJ133783	11	Konrad *et al.*, 1999
Rap 1	H. sapiens TRF2-interacting telomeric RAP1 protein	NM_204468	4	Tan *et al.*, 2003
Rap 1	G. gallus TRF2-interacting telomeric RAP1 protein (RAP1) mRNA	AY083908	11	Tan *et al.*, 2003
Pot 1	H. sapiens protection of telomeres 1	NM_015450	7q31.33	Bauman and Cech, 2001
Pot 1	G. gallus POT1 single-strand telomeric DNA-binding protein	AY555718	1	Wei and Price, 2004
WRN	H. sapiens Werner Syndrome (WRN) protein	NM_000553	8p12-p11.2	Gray *et al.*, 1997
WRN	G. gallus Werner Syndrome protein (WRN)	NM_001012888	4	Caldwell *et al.*, 2005
BLM	H. spaiens Bloom Syndrome (BLM) protein	NM_000057	15q26.1	Ellis *et al.*, 1995
BLM	G. gallus Bloom Syndrome (BLM) protein	NM_001007087	10	Caldwell *et al.*, 2005
c-myc	H. sapiens c-myc oncogene	V00568	8q24	Watt *et al.*, 1983
c-myc	G. gallus c-myc oncogene	X68073	2	Harris *et al.*, 1992

(Taylor and Delany, 2000; Forsyth *et al.*, 2002; Swanberg and Delany 2003; Delany *et al.*, 2003).

Unlike mouse fibroblasts, both chicken and human primary fibroblast cells are generally refractory to spontaneous immortalization (Lima and Macieira-Coelho, 1972; Lima *et al.*, 1972; Macieira-Coelho and Azzarone, 1988; Prowse and Greider, 1995). In addition, critically short human telomeres induce senescence either by activating p53 or by inducing the p16/RB pathway, and suppression of both pathways is required to suppress senescence of aged human cells. In mouse, the p16/RB response to telomere dysfunction is not active (Smogorzewska and de Lange, 2002). In contrast, the senescence pathways of chicken and human fibroblast systems thus far seem to share more similarities than differences (Kim *et al.*, 2002); see Table 29.2. For an

TABLE 29.2
Telomerase activity, telomere shortening and ease of immortalization in vertebrate model systems

Mouse	Human	Chicken
Telomerase activity in somatic cells	No telomerase activity in most somatic cells	No telomerase activity in most somatic cells
No division-dependent telomere shortening	Division-dependent telomere shortening	Division-dependent telomere shortening
Fibroblasts spontaneously immortalize	Fibroblasts refractory to spontaneous immortalization	Fibroblasts refractory to spontaneous immortalization

excellent review of the developmental regulation of telomerase activity in human, mouse, chicken and flowering plants, see Forsyth *et al.* (2002).

Features of the Chicken Genome Relevant to the Study of Aging

The chicken karyotype consists of 39 pairs of chromosomes, which is typical of most avian species. The genome is organized as eight pairs of cytologically distinct macrochromosomes, the Z and W sex chromosomes and thirty pairs of small cytologically indistinguishable microchromosomes (ICSGS, 2004). As in other vertebrates, chicken telomeres consist of a highly conserved hexanucleotide repeat, 5′ $TTAGGG_{(n)}$ 3′. The cytogenetic features of the telomere repeat were first described in chicken by Nanda and Schmid (1994). Molecular features of telomeric DNA in the chicken genome were described in 2000 (Delany *et al.*). Although the avian genome is one-third the size of the human genome (1.25 pg versus 3 pg/haploid cell), the amount of telomeric DNA sequence is five to ten times more abundant in birds than in humans (Delany *et al.*, 2000; Nanda *et al.*, 2002). Higher telomere repeat content in the chicken is likely due to the high number of chromosome ends ($2n = 78$ or 156 chromosome termini), the load of interstitial telomeric DNA and the presence of an unusual category of ultra-long telomeric arrays (see Figure 29.1).

Telomeric DNA in the chicken can be categorized into three main array size classes. Class I telomere repeats are 0.5–10 kb in length and exhibit discrete and genotype-specific banding patterns. Class I repeats are interstitially located and show no evidence of telomere shortening. Class II repeats are 10–40 kb and appear on Southern blots as the typical overlapping smear of TRFs; Class II arrays show evidence of terminal location based on digestion by *Bal* 31 and exhibit division-dependent shortening in somatic tissues. Class III telomeres are hundreds of kilobases in size and range to 3 megabases. Shortening of these arrays has not been established because of the inability to resolve changes of 100s of nucleotides (typical telomere erosion) in the context

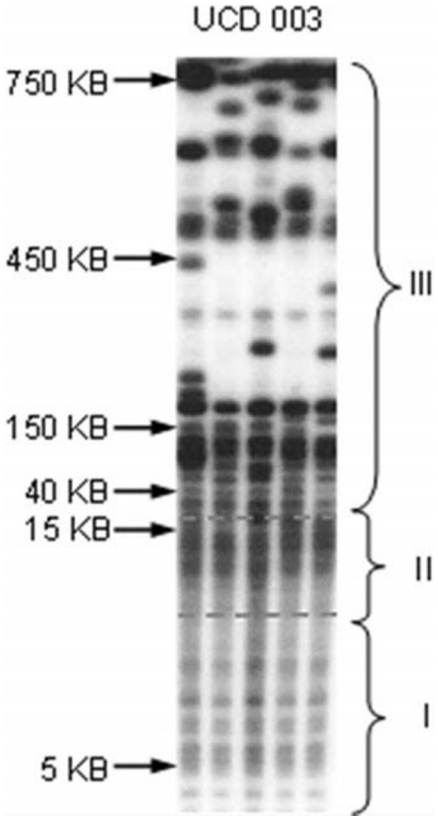

Figure 29.1. Image of pulse-field gel showing chicken Class I, II and III telomere arrays. Class II arrays are analyzed for telomere shortening.

of 100s to 1000s of kilobases of the Class III arrays (Delany *et al.*, 2000). In order to resolve Class III arrays on a gel, special pulse field gel electrophoresis parameters are required (Delany *et al.*, 2000).

Not all avian species exhibit the Class III arrays (Delany *et al.*, 2000; Nanda *et al.*, 2002). Current models suggest that the Class III arrays of the chicken map to a subset of microchromosomes, perhaps serving to protect these small genetic elements from erosion and/or contributing to high microchromosome recombination rates (Delany *et al.*, 2000; Delany

et al., 2003). It is important to note that the existence of megabase telomere arrays in chicken does not diminish the power of the chicken as a model for division-dependent telomere shortening as it appears to be the shortest telomere or the unprotected telomere which triggers genome instability (Hemann *et al*., 2001; Karlseder *et al*., 2002).

Telomerase activity and telomere-shortening profiles in avian cells *in vivo* and *in vitro* mirror what is observed in human cells. Telomerase activity is developmentally regulated *in vivo* with high levels of telomerase in early-stage chicken embryos (preblastula through neurula) and during organogenesis all organs surveyed up to 10 days of embryonation (E10) followed by down-regulation for most somatic tissues. Constitutive telomerase activity continues for ''renewable'' tissues, including intestine, spleen, and organs or cells of the reproductive system. An average decrease of 3.2 kb in telomere length was observed from the early embryo to the adult (Taylor and Delany, 2000). *In vitro* observations include absence of telomerase from nontransformed primary cells (CEFs) contrasted with telomerase activity in cultured blastodermal cells, cES cells and in every transformed avian cell type surveyed to date (Table 29.3).

As measured by mean TRF, the *in vitro* rate of telomere shortening observed in Class II arrays in CEFs is approximately 50 bp of telomeric DNA per population doubling. Yet calculation of percent telomeric DNA at representative passages revealed that an average of 63% of the telomeric DNA was eroded in CEFs by senescence. The greatest loss of telomeric DNA occurred precipitously in later passages. These data suggest two mechanisms of telomere shortening: (1) telomere attrition due to the end-replication problem and (2) catastrophic erosion preceding culture arrest (Swanberg and Delany, 2003).

TABLE 29.3
Telomerase Positive Transformed Avian Cell Lines
(Adapted from Swanberg and Delany, 2003)

Cell name	Description
RP-19	Turkey B cell
DT40	Chicken B cell (bursal lymphoma)
RP-9	Chicken B cell (bursal lymphoma)
MSB-1	Chicken T cell (spleen tumor cells in vitro)
MQ-NCSU	Chicken macrophage (peripheral blood)
QT6	Quail fibroblast (fibrosarcoma)
QT35	Quail fibroblast (fibrosarcoma)
LMH & LMH/2A	Chicken hepatocyte (hepatocellular carcinoma)

Tools for Utilizing the Chicken in Aging Studies

A variety of techniques for the study of telomere biology are available, including telomere terminal restriction fragment (TRF) analysis, fluorescence *in situ* hybridization (FISH), variations of polymerase chain reaction (PCR), and the telomere repeat amplification protocol (TRAP). For an excellent summary of selected methods utilized to measure telomere length, see Nakagawa *et al*., (2004). A list of references pertaining to techniques used in the study of telomere biology is contained in Table 29.4.

TABLE 29.4
References for the study of Avian Telomere Biology

Telomere cytogenetics
Nanda and Schmid, 1994
Nanda *et al*., 2002
Delany *et al*., 2000
Replicative senescence in chicken cell culture
Lima *et al*., 1972
Lima and Macieira-Coelho, 1972
Measuring telomeres
Harley *et al*., 1990
Nakagawa *et al*., 2004
Telomere shortening in birds
Talyor and Delany, 2000
Delany *et al*., 2003
Swanberg and Delany, 2003
Telomeres as tool for age determination
Vleck *et al*., 2003
Haussmann *et al*., 2003
Hall *et al*., 2004
TRAP assay
Kim *et al*., 1994
Saldanha *et al*., 2003
Telomerase in birds
Talyor and Delany, 2000
Delany *et al*., 2000
Swanberg and Delany, 2003
Venkatesan and Price, 1998
Haussman *et al*., 2004
Gene expression patterns: telomere maintenance pathways
Swanberg *et al*., 2004
Swanberg and Delany, 2005

(*Continued*)

TABLE 29.4
Continued

Quantitative PCR
Bustin, 2004
Gain- and loss-of-function techniques in chick embryo
Krull, 2004 (electroporation)
Bourikas and Stoeckli, 2003 (RNAi)
Pekarik *et al.*, 2003 (RNAi)
Sato *et al.*, 2004 (RNAi)
Kos *et al.*, 2003 (morpholinos)
Chicken genome sequence and genomic resources
ICGSC, 2004
Antin and Konieczka, 2005
Dequeant and Pourquie, 2005

TELOMERE TERMINAL RESTRICTION FRAGMENT ANALYSIS

First described in Harley *et al.*, 1990, telomere terminal restriction fragment analysis establishes mean telomere length in a tissue or cell sample or percent of telomeric DNA present in one sample relative to another. To measure mean telomere length, genomic DNA is first digested with a restriction enzyme or a cocktail of restriction enzymes followed by electrophoretic separation through an agarose gel. It is essential that DNA concentration be equivalent in each lane. The gel is Southern blotted and hybridized to a TTAGGG(n) probe labeled with a radionuclide or a fluorochrome producing a smear of fragments. Densitometry readings taken at a number of locations along the smear are summed and averaged. Mean telomere length is defined as $\sum(OD_i)/\sum(OD_i/L_i)$ where OD_i is the densitometer output and L_i is the length of the DNA at position i. Sums are calculated over the range of lengths covered by the smear of TTAGGG-hybridized DNA (Harley *et al.*, 1990, Swanberg and Delany, 2003).

In order to measure percent telomeric DNA present in one sample relative to a calibrator sample (Harley *et al.*, 1990), DNA is restricted, separated by gel electrophoresis, Southern blotted, and hybridized as with the determination of mean TRF length. However, rather than taking densitometry readings at discrete locations along the length of the smear of telomeric DNA, total telomeric DNA is measured by calculating the total integrated signal $\left(\sum OD_i\right)$ over the same range of fragment sizes used for mean TRF analysis. Total integrated signal in this range is measured in each lane of any given gel, and results are expressed as a percentage of the signal from the earliest passage (Harley *et al.*, 1990; Swanberg and Delany, 2003). The measurement of TRFs reveals a high degree of variability within cell lines prepared from single embryos of a highly inbred line, and mean TRF measurements are also subject to variability resulting from drift in the subpopulations within a culture. Therefore it is advisable to assay using more than one method to obtain a biologically relevant picture of telomere attrition or erosion (Swanberg and Delany, 2003).

FISH

Telomere arrays have been examined in a wide sampling of avian species, including chicken, using fluorescence *in situ* hybridization (FISH) (Nanda and Schmid, 1994; Nanda *et al.*, 2002). While the Nanda study was not quantitative, the existence of large telomere arrays in birds was quite apparent using traditional FISH techniques. Telomere quantitative fluorescence *in situ* hybridization (telomere Q-FISH), a variation of this method, has been utilized effectively in several organisms. Using Q-FISH, telomere length is expressed as a ratio of telomere fluorescence in cells that have undergone erosion to telomere fluorescence in cells in the same tissue section with intact telomeres. The inherent disadvantage of Q-FISH is that only a small subset of telomeres can be examined at any one time relative to the bulk methods (e.g., TRF analysis) (Nakagawa *et al.*, 2004 and references therein).

PCR-BASED METHODS FOR TELOMERE LENGTH MEASUREMENT

A technique that addresses some of the limitations of Q-FISH is single telomere length analysis (STELA). Using STELA, a 20-mer noncomplementary oligonucleotide with a TTAGGG tail is linked to the G-rich 3′ overhang of the telomere. The TTAGGG tail is then ligated to the complementary 5′ strand of the telomere. PCR is performed using one primer for the linked oligo and a second primer recognizing unique subtelomeric sequence. Use of this technique requires identification of subtelomeric sequences, which has not yet been accomplished in avian species, but should be possible in chicken now that the genome is sequenced (Nakagawa *et al.*, 2004 and references therein). Edges of telomeric DNA were identified in the draft sequence for the macrochromosomes (ICGSC, 2004, see supplementary information).

A second PCR-based technique that can be used to compare the abundance of telomere repeats is quantitative real-time PCR (Q-PCR). This technique quantifies the fold-difference between telomere-repeat copy number in an experimental sample compared to a reference DNA sample. Disadvantages of this method are that it does not determine absolute telomere length and that interstitial telomere sequences, present in avian species, will be measured as well as terminal repeats (Nakagawa *et al.*, 2004 and references therein). This should not be a problem if telomere shortening is being measured, because the number of interstitial repeats should not change relative to terminal repeats unless dramatic genome

reorganization such as a breakage-fusion-bridge cycle is occurring.

TRAP ASSAY

The telomerase repeat amplification protocol (TRAP) assay, first described by Kim *et al*. (1994), relies upon primer extension of an oligonucletide by telomerase. Cells are lysed and cellular protein extracts are incubated with an oligonucletide to which a series of TTAGGG repeats will be added when telomerase is present in the cell extract. Variations of the TRAP assay exist, including radioactive or nonradioactive gel-based detection, ELISA-based detection and semiquantitative or quantitative protocols. For an excellent review of the TRAP assay and many of its iterations, see Saldanha *et al*. (2003).

GENE EXPRESSION ANALYSIS

Real-time fluorescence-based PCR and RT-PCR have emerged as powerful methods for examining gene expression patterns in many contexts. In traditional PCR, an amplicon which accumulates after a predetermined number of cycles is analyzed by gel electrophoresis. In real-time PCR, reactions are characterized by the PCR cycle at which amplification of a target molecule is first detected by release of a fluorescent signal in real time. The greater the quantity of the target molecule in the reaction mix, the earlier a significant increase in fluorescence will be measured. Quantitation is accomplished with reference to a threshold cycle, (C_t), defined as the fractional cycle number at which fluorescence, generated by the increase in PCR product, exceeds a set threshold above the baseline. For an excellent treatise on fluorescence-based real-time PCR, refer to Bustin *A–Z of Quantitative PCR* (2004).

Recently, real-time quantitative TaqMan PCR was utilized to look at expression of genes involved in chicken telomere maintenance pathways. Chicken primers and fluorescent probes were developed for seven target genes (tankyrase 1, tankyrase 2, TRF1, TRF2, cTERT, cTR and c-myc) as well as for three housekeeping genes for normalization purposes. In cell culture, chicken GAPDH mRNA levels were found to show the least standard deviation for all samples examined, and therefore GAPDH values were used to normalize the target gene values.

Analysis of mRNA expression patterns of the target genes in CEFs, DT40, the gastrula embryo and cES cells revealed up-regulation of tankyrase 2, TRF1, TRF2, c-myc, cTERT and cTR in DT40 cells, with c-myc levels up-regulated 184-fold in DT40 relative to the gastrula and 282-fold in DT40 relative to CEFs and cES cells. Telomerase holoenzyme components (cTERT and cTR) were present, although at low levels, in CEFs and were up-regulated in DT40, cES cells and the gastrula relative to CEFs. Down-regulation of TRF1, c-myc, cTERT and cTR appeared to be a feature of senescing CEFs that had survived an average of 30.5 PD (Swanberg *et al*., 2004; Swanberg and Delany, 2005). For a detailed discussion of these expression patterns as well as primer and probe sets, for target and housekeeping genes, see Swanberg *et al*. (2004) and Swanberg and Delany (2005).

ELECTROPORATION, RNAi AND MORPHOLINOS

One of the requirements for a good model system is the ability to do gain- and loss-of-function experiments. Techniques exist to perform such experiments in chicken. A number of investigators utilize electroporation to introduce exogenous DNA into the chicken embryo *in ovo* (Muramatsu *et al*., 1997). For an excellent review of electroporation techniques *in ovo*, see Krull (2004). Loss-of-function experiments can be conducted by introducing short, interfering RNAs (siRNAs) or morpholinos into the chick embryo.

Double-stranded RNA-mediated interference (RNAi), a naturally occurring mechanism which results in the silencing of gene expression, has become a very powerful tool for experimental gene suppression in a number of organisms. The phenotypes observed with RNAi silencing of gene expression range from knockdown to knockout (Agrawal *et al*., 2003). RNAi was successfully exploited in chicken (*in ovo*) for gene silencing (Bourikas and Stoeckli, 2003; Pekarik *et al*., 2003; Krull 2004; Sato *et al*., 2004). In addition to siRNAs, RNAi morpholinos were used in loss-of-function studies in chicken. For example, Sheng *et al*. (2003) used morpholino oligonucleotides to knock down expression of genes in the future neural plate of the chicken embryo.

GENOMIC TOOLS

Information regarding web-based tools for sequence and bioinformatics analysis of avian species, BAC and cDNA libraries, chicken gene chips and a number of other websites of interest to researchers are contained in Table 29.5. For further detail on cDNA arrays for chicken gene expression analysis, see Burnside *et al*. (2005). Tutorials oriented toward the biologist new to bioinformatics can be found in Antin and Konieczka (2005). Both Antin and Konieczka (2005) and Dequeant and Pourquie (2005) describe additional resources for the study of chicken genomics.

TELOMERES AS A TOOL FOR AGE DETERMINATION IN BIRDS

Estimating age in unmarked bird populations is of primary interest to many disciplines. The relationship between telomere shortening and chronological age was studied recently by determination of the telomere rate of change (TROC) or ''telomere clock'' in a number of bird species. Measuring the length of TRFs in DNA from erythrocytes and plotting mean telomere length against the maximum lifespan in years for each species, a correlation between TROC and lifespan was indicated.

TABLE 29.5
Internet resources for researchers in avian telomere biology

Genomic Resources	*URL*
ArkDB Chicken database	http://www.thearkdb.org/browser?species=chicken
Avian Sciences Net-Purdue	http://ag.ansc.purdue.edu/poultry/
AvianNET	http://www.chicken-genome.org
BBSRC ChickEST Database	http://chick.umist.ac.uk/
Chick FPC Database-Wageningen University	http://www.animalsciences.nl/ChickFPC/
ChickBASE	http://www.genome.iastate.educ/chickmap/dbase.html
Chicken genome mapping database of the Animal Sciences Group (Wageningen Universtiy)	https://acedb.asg.wur.nl/
Chicken Genome White Paper	http://genome.wustl.edu/projects/chicken/Chicken_Genome.pdf
Chicken SAGE Website	http://www.cgmc.univ-lyon1.fr/Grandrillon/chicken_SAGE.php
CHORI-261 Chicken BAC Library, Children's Hospital Oakland Research Institute	http://bacpac.chori.org/chicken261.htm
Ensemble Chicken Genome Browser	http://www.ensembl.org/Gallus_gallus/
Gallus gallus EST and in situ hybridization analysis database	http://geisha.biosci.arizona.edu/
Gallus gallus Trace Archive	http://www.ncbi.nih.gov/Traces/trace.cgi
GENEfinder Genomic Resources (Red Jungle and Chicken BAC Libraries) TAMU	http://hbz.tamu.edu/bacindex.html
NCBI Clone Registry	http://www.ncbi.nlm.nih.gov/genome/clone/query.cgi?EXPR=chicken
NCBI's compendium of chicken genomic resources	http://www.ncbi.nlm.nih.gov/projects/genome/guide/chicken/
NetVet	http://netvet.wustl.edu/birds.htm
Sanger Institute Chicken Genome Sequencing Project	http://www.sanger.ac.uk/Projects/G_gallus/
UD Chicken EST Database	http://www.chickest.udel.edu/
US Poultry Genome Website	http://poultry.mph.msu.edu/
Chicken Genome Array (Affymetrix)	http://www.affymetrix.com/products/arrays/specific/chicken.affx
Stocks, Lines and Cell Lines	
Poultry and Avian Research Resources: Living Stock Populations	http://animalscience.ucdavis.edu/AvianResources/
American Type Culture Collection (ATCC)	http://www.atcc.org
Husbandry	
CSREES – USDA Animal Breeding, Genetic and Genomics	http://www.csrees.usda.gov/ProgView.cfm?prnum=4030
USDA Animal Welfare Information Center	http://www.nal.usda.gov/awic/
USDA Bibliography on Poultry Production	http://www.nal.usda.gov/afsic/AFSIC_pubs/livestock/srb0406ch6.pdf
USDA Agricultural Research Service-Poultry Research Publications	http://www.lpsi.barc.usda.gov/gblab/research/poultryindex.html
USDA Housing, Husbandry, and Welfare of Poultry	http://netvet.wustl.edu/species/birds/QB9415.HTM
Poultry Science Association	http://www.poultryscience.org/

In most of the species studied, telomeres appeared to shorten more slowly in long-lived birds than short-lived birds. Interestingly, in a particularly long-lived bird, Leach's storm petrel, telomeres did not shorten with age, but lengthened (Vleck *et al*., 2003, Haussmann *et al*., 2003).

In another study examining DNA from erythrocytes, it was found that while telomere length in blood cells declined between the chick stage and the adult in two species of long-lived seabirds, telomere length in adults was not related to age. This study cautioned that rates of telomere loss were not constant with age and that there was a great deal of interindividual variation in the magnitude of telomere loss (Hall *et al*., 2004). It should be noted that avian erythrocytes are the product of erythroid progenitor cells capable of extended self-renewal (Beug *et al*., 1994) and therefore are likely to possess a significantly different telomere-length maintenance pathway than the majority of somatic cells whose telomeres typically demonstrate division-dependent shortening. It would not, therefore, be surprising to find that telomere shortening profiles in this renewable cell population would bear a greater resemblance to the profiles of other renewable tissues than to the telomeres of nonrenewable cell populations such as fibroblasts.

Research Resources: Stocks and Lines, Cells and Cell Lines

A variety of avian stocks and lines are available to the investigator of telomere biology. Genetic stocks and mutant lines are listed in an Avian Stocks Database linked to the Poultry and Avian Research Resources: Living Stock Populations website of the Animal Science Department, University of California, Davis (see Table 29.5 for URL). In addition, a selection of transformed and nontransformed avian cells and cell lines are available through the American Type Culture Collection (see Table 29.5 for URL). Protocols for primary culture of isolated chicken tissues can be found in Fresheny, *Culture of Animal Cells: A Manual of Basic Technique* (2000).

Husbandry

A number of excellent resources on basic chicken biology and husbandry are available including Sturkie's *Avian Physiology* (2000) and Scanes' *Poultry Science* (2004). In addition, the United States Department of Agriculture and several other agencies or associations provide both web-based and written materials on poultry husbandry and animal welfare. Table 29.5 contains web-based resources on poultry husbandry and related topics.

Conclusions

The study of telomere biology and telomere maintenance pathways has provided and will continue to provide a great deal of insight into the processes of replicative senescence, the relationship between cellular senescence and organismal aging, the genesis of cancer, and the regenerative potential of embryonic stem cells. Use of *in vivo* and *in vitro* avian systems to facilitate research in these fields can only add to our body of knowledge. With the 6.6X draft sequence of the chicken genome now available, the chicken is a much more powerful model.

Investigation of telomere maintenance pathways in the chicken and other birds establishes, among other things, that nonrenewable cells and tissues exhibit little or no telomerase activity accompanied by division-dependent telomere shortening; that embryonic cells and tissues as well as transformed cells exhibit high levels of telomerase; and that many telomere-associated genes are expressed differentially in pluripotent, differentiated and transformed cell systems, much as is seen in human systems. TERT and TR genes are transcribed in at least one telomerase-negative cell type, which suggests that the regulation of telomerase activity is more complex than merely switching the genes for telomerase enzyme components on and off. While telomere shortening profiles are unlikely to be the equivalent of rings on a tree for the determination of chronological age, comparisons of telomere status in pluripotent vs. differentiated, transformed vs. nontransformed and early passage vs. senescent cells are informative. Considerable work is necessary to fill in gaps, but the chicken model for telomere biology offers the opportunity to study a vertebrate system free from many of the issues inherent in the murine model. Chickens, therefore, have the potential to become the new "lab rat" for aging research.

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