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Differential expression of genes associated with telomere length homeostasis and oncogenesis in an avian model

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Abstract

Telomere-binding proteins, their interaction partners and transcription factors play a prominent role in telomere maintenance and telomerase activation. We examined mRNA expression levels of tankyrase 1 and 2, TRF1 and 2, c-myc, TERT and TR in *Gallus domesticus*, the domestic chicken, by quantitative real-time PCR, establishing expression profiles for three contrasting cell systems: the pluripotent gastrula, differentiated embryo fibroblasts and transformed DT40 cells. All seven genes were up-regulated in DT40 cells compared to telomerase-negative CEFs and a majority of the genes were also up-regulated in the gastrula relative to CEFs. Surprisingly, we found TERT and TR transcripts in CEFs, albeit at low levels. TRF1 was down-regulated in the six CEF cultures by the time of culture growth arrest. A marked increase in the TRF2:TRF1 ratio occurred at or near senescence in all of the CEF cultures studied, with the most elevated ratio found in a short-lived culture in which TRF1 mRNA levels decreased two-fold and TRF2 levels increased 21-fold. This culture also showed highly reduced, degraded telomeres by Southern blot analysis. These data suggest that genes involved in telomere maintenance and telomerase induction are expressed differentially in pluripotent, differentiated and transformed cell systems.
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1. Introduction

Telomeres, the ends of eukaryote linear chromosomes consisting of tandem arrays of telomeric repeats, protect the genome from degradation. In vertebrates, telomeres are composed of thousands of duplex DNA repeats of the sequence 5′ TTAGGG 3′, with the G-rich strand extending as a 3′ overhang. A major part of the vertebrate telomere is packaged in closely spaced nucleosomes (Blackburn, 2001). However, the 3′ G-rich overhang assumes a terminal loop configuration (t-loop) displacing one of the duplex strands and forming a related structure (D-loop). The D-loop t-loop is stabilized by telomere-binding proteins and their interaction partners (Wei and Price, 2003; Greider, 1999; Griffith et al., 1999).

The end-replication problem, the incomplete replication of the 5′ end of each daughter strand, results in progressive shortening of telomeres leading to genome instability (Levy et al., 1992). Telomerase provides a means to replace telomere repeats which are lost during replication as a result of the inability of DNA polymerase to replicate to the end of a linear chromosome. Telomerase activity not only maintains the telomeres of proliferating cells but is implicated in the process of cellular immortalization and oncogenesis (Greider and Blackburn, 1989). Telomerase RNA, TR, contains the template for addition of telomeric repeats (Greider and Blackburn, 1989) and is generally believed to be constitutively expressed (Yi et al., 2001). Telomerase reverse transcriptase, TERT, the component which catalyzes the addition of these repeats to the parent-strand chromosome end, has been described as the rate-limiting molecule in the assembly of the telomerase holoenzyme (Zou et al., 2005). Transfection of non-transformed, telomerase-negative human cells with a vector encoding the human telomerase catalytic subunit resulted in the elongation of telomeres and extension of the lifespan of the cells which would otherwise have undergone replicative senescence (Bodnar et al., 1998).

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Telomere-associated proteins involved in the regulation of telomere length include telomere-repeat-binding factors 1 and 2 (TRF1 and 2) each of which binds as a homodimer to double-stranded telomeric DNA or also, in the case of TRF2, as an oligomer (Wei and Price, 2003). TRF1 induces bending, looping and pairing of double-stranded telomeric DNA (Smogorzewska et al., 2000; Bianchi et al., 1997) and may induce shortening of telomeres by sequestering the 3′ overhang from telomerase (Van Steensel and de Lange, 1997). TRF2 is described both as protective of telomeres (Karlseder, 2003) and as a negative regulator of telomere length (Stansel et al., 2001; Smogorzewska et al., 2000). Long-term expression of both TRF1 and TRF2 by stable transfection or overexpression of TRF1 or TRF2 produces a progressive shortening of telomeres (Ohki and Ishikawa, 2004; Karlseder et al., 2002; Smogorzewska et al., 2000; Van Steensel and de Lange, 1997). In contrast, expression of a dominant negative TRF1 mutant which does not bind telomeric DNA results in telomere elongation (Ohki and Ishikawa, 2004; Van Steensel and de Lange, 1997). New studies suggest that TRF2 and TRF1 function cooperatively as both proteins can be found linked to a third protein, TIN2, which may stabilize the binding of TRF1 and TRF2 to the telomere (Ye et al., 2004; Houghtaling et al., 2004). Interaction partners of TRF1 include the tankyrases (De Rycker et al., 2003). The binding of tankyrase 1 or 2 to TRF1, resulting in the ADP-ribosylation of TRF1, may attenuate the affinity of TRF1 for telomeric DNA. Consistent with this relationship, overexpression of tankyrase 1 results in the removal of TRF1 from the telomeres followed by telomere elongation (Smogorzewska and de Lange, 2004).

In addition to the telomere-binding proteins and their interaction partners, other proteins play a role in telomere length regulation including c-myc, an oncogenic transcription factor known to regulate cell proliferation, differentiation and apoptosis as well as cell size (Piedra et al., 2002). The expression of c-myc is down-regulated in quiescent and differentiated cells and, in fact, c-myc down-regulation might be a necessary prerequisite to differentiation (Baker et al., 1994; Skerka et al., 1993). Recent research suggests that c-myc re-activates telomerase in transformed cells by inducing expression of its catalytic subunit TERT (Wu et al., 1999).

The chicken has long been recognized as a premier model organism in developmental biology (Antin et al., 2004 and associated papers) and shows promise as a model for research in the biology of aging, including telomere biology. Somatic cells of the domestic chicken, *Gallus domesticus*, share the following telomere-related features with human somatic cells: down-regulation of telomerase activity, division-dependent telomere shortening both in vivo and in vitro and re-emergence of telomerase in oncogenic cells (Swanberg and Delany, 2003; Delany et al., 2003; Taylor and Delany, 2000). Interestingly, both human and chicken cells are refractory to transformation. In contrast, mouse somatic cells exhibit constitutive telomerase activity, show no division-dependent shortening of telomeres and are readily amenable to transformation (Swanberg and Delany, 2003; Forsyth et al., 2002). With the similarity of the chicken and human telomere clocks and the array of new genomic tools including the chicken genome draft $6.6 \times$ sequence (Antin and Konieczka, 2005), the chicken is poised to emerge as a powerful new model in aging research.

Orthologs of tankyrase 1 and 2, TRF1 and 2, TERT and TR are described for the domestic chicken, *Gallus domesticus* (Delany and Daniels, 2004; De Rycker et al., 2003; Delany and Daniels, 2003; Chen et al., 2000; Konrad et al., 1999) and c-myc was first discovered in chicken (Hayward et al., 1981). To further our knowledge of chicken telomere biology, we examined mRNA expression of tankyrases 1 and 2, TRF1 and 2, c-myc, TERT and TR by quantitative real-time PCR in the chicken model. Expression patterns were studied in six chicken embryo fibroblast (CEF) cultures with different lifespan phenotypes as well as in the gastrula and DT40 cells. In both the telomerase-positive gastrula and DT40 cells, the genes were more abundantly transcribed than in most of the telomerase-negative CEF samples. Notable differences in transcription patterns, consistent with the proliferative potential of each cell type, were identified.

2. Materials and methods

2.1. Cell culture

CEFs were isolated from six E11 (11 days of embryogenesis) embryos from UCD 003, a highly inbred chicken line (Pisenti et al., 1999). Individual cultures (unsynchronized) were derived from single embryos and maintained in DMEM with L-glutamine, 10% FBS, and 5% penicillin-streptomycin in a humidified 95% air, 5% CO_2 atmosphere as previously described (Swanberg and Delany, 2003). The cultures were passaged when they reached 80–90% confluence and split 1:3 or 1:4 until culture arrest. Population doubling (PD) was determined for each passage using the following equation:

$$\text{population doubling} = \frac{\log N_{\text{t}} - \log N}{\log 2}$$

With N the number of cells seeded and N_{t} the number of viable cells at the end of the passage (Patterson, 1979; Venkatesan and Price, 1998). Senescence was determined by growth dynamics, cellular morphology and by a β-galactosidase assay (Dimri et al., 1995). Senescence staining was performed using the Senescence β-galactosidase Staining Kit (Cell Signaling Technology). Cultures were deemed senescent when >90% of the cells were positive for β-galactosidase (Swanberg and Delany, 2003).

Four cultures lived for an average of 30.4 PD (range from 29 to 32 PD), one short-lived culture reached senescence at 24 PD and one culture exhibited a longer lifespan of 36 PD.