Cardiac Stem Cell and Myocyte Aging, Heart Failure, and Insulin-Like Growth Factor-1 Overexpression

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Abstract—To determine whether cellular aging leads to a cardiomyopathy and heart failure, markers of cellular senescence, cell death, telomerase activity, telomere integrity, and cell regeneration were measured in myocytes of aging wild-type mice (WT). These parameters were similarly studied in insulin-like growth factor-1 (IGF-1) transgenic mice (TG) because IGF-1 promotes cell growth and survival and may delay cellular aging. Importantly, the consequences of aging on cardiac stem cell (CSC) growth and senescence were evaluated. Gene products implicated in growth arrest and senescence, such as $p27^{Kip1}$, p53, $p16^{INK4a}$, and $p19^{ARF}$, were detected in myocytes of young WT mice, and their expression increased with age. IGF-1 attenuated the levels of these proteins at all ages. Telomerase activity decreased in aging WT myocytes but increased in TG, paralleling the changes in Akt phosphorylation. Reduction in nuclear phospho-Akt and telomerase resulted in telomere shortening and uncapping in WT myocytes. Senescence and death of CSCs increased with age in WT impairing the growth and turnover of cells in the heart. DNA damage and myocyte death exceeded cell formation in old WT, leading to a decreased number of myocytes and heart failure. This did not occur in TG in which CSC-mediated myocyte regeneration compensated for the extent of cell death preventing ventricular dysfunction. IGF-1 enhanced nuclear phospho-Akt and telomerase delaying cellular aging and death. The differential response of TG mice to chronological age may result from preservation of functional CSCs undergoing myocyte commitment. In conclusion, senescence of CSCs and myocytes conditions the development of an aging myopathy. **(*Circ Res*. 2004;94:514-524.)**

Key Words: telomerase ■ telomere dysfunction ■ cellular senescence

The accepted but never proven paradigm is that the heart is a postmitotic organ characterized by a predetermined number of myocytes, which is defined shortly after birth and is preserved throughout life till death of the organism.[1] According to this view, age of cardiomyocytes corresponds to the age of the organ and organism, ie, without exception, cellular, organ, and organism age coincide. Myocytes must age at the same pace and, at any given time, the heart should be composed of a homogeneous population of myocytes of identical age. Therefore, myocardial aging has been interpreted as a time-dependent biological process that interacts with ischemic heart disease, hypertension, diabetes, and other pathological conditions, which together define the clinical phenotype.[2] The possibility that cardiac aging is an independent determinant of morbidity and mortality has faced opposition and emphasis has been placed on age-associated changes, which increase the chances of cardiovascular events in the elderly. Treatment of cardiac diseases in old patients has resulted in a prolongation of average lifespan. However, maximum lifespan has not increased in the last 70 years,[3] suggesting that cellular aging may play a more important role than generally expected.

Several lines of evidence have been obtained in favor of the regeneration of the adult and aged myocardium[4] through the activation of cardiac stem cells (CSCs).[5,6] A mitotic clock regulates cellular lifespan that is not related to organ and organism age and lifespan,[7] raising the possibility that CSC aging conditions myocyte aging and the development of an aging myopathy. Although this may be seen as an oversimplification of a complex problem, myocardial aging may result from senescence of myocytes due to attenuation in their turnover dictated by CSC aging and the inevitable accumulation of older cells. Therefore, the hypothesis being tested is whether an imbalance between myocyte growth and death induced by alterations in CSC function occurs with age resulting in a decompensated myopathy. Conversely, growth

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factors enhancing cell replication and attenuating CSC and myocyte senescence may delay the appearance of a cardiomyopathic old heart.

Materials and Methods

An expanded Materials and Methods can be found in an online data supplement available at http://circres.ahajournals.org.

Results

Myocyte Aging

Cellular aging is characterized by the expression of nuclear proteins involved in cell cycle inhibition and irreversible growth arrest. Growth factors promoting cell division may interfere with cellular senescence.[8] A most likely candidate is insulin-like growth factor-1 (IGF-1) whose overexpression in myocytes potentiates new myocyte formation in the adult heart.[9] Significant differences may exist in the phenotype of aging myocytes in wild-type (WT) and IGF-1 transgenic (TG) homozygous mice at 4, 10 to 12, and 20 to 22 months of age. This likelihood would strengthen the notion that senescence of parenchymal cells results in an aging myopathy, distinct from age-associated diseases.[2]

During aging, fibroblasts[10] are arrested at G0-G1 by transient expression of $p21^{Cip1}$ followed by a temporary upregulation of $p27^{Kip1}$ and then by persistent elevation of $p16^{INK4a}$. A comparable role for $p19^{ARF}$ and p53 was recognized in cellular aging.[11] The regulation of $p21^{Cip1}$ can be p53-dependent or -independent[12] and $p19^{ARF}$ increases p53 quantity and activity[13] by decreasing Mdm2 expression.

A similar pattern of gene expression was found in vivo in myocytes from aging WT: $p21^{Cip1}$ decreased from 4 to 20 to 22 months, whereas $p27^{Kip1}$, $p16^{INK4a}$, and $p19^{ARF}$ increased. The increase in $p19^{ARF}$ was coupled with an upregulation of total and free p53 protein, which was paralleled by a decrease in the formation of Mdm2-p53 inactive complexes (Figure 1). IGF-1 opposed these age-dependent alterations in myocytes. With respect to WT, $p21^{Cip1}$ was detected in lower quantities in TG myocytes at 4 and 10 to 12 months and was undetectable at 20 to 22 months. $p27^{Kip1}$, $p16^{INK4a}$, and $p19^{ARF}$ were absent at 4 months and present in modest levels at 10 to 12 and 20 to 22 months. p53 increased moderately with age and Mdm2-p53 complexes remained high in TG myocytes.

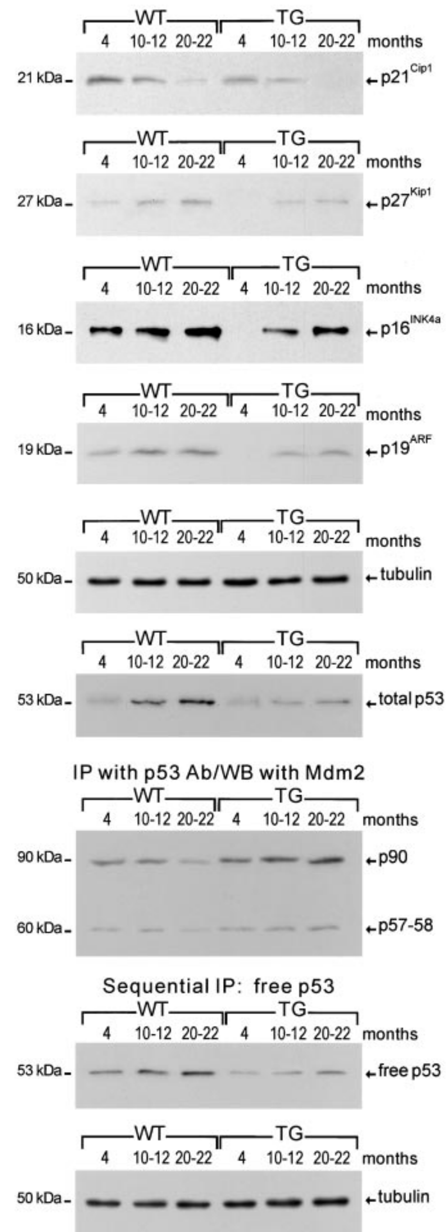

Figure 1. Inhibitors of myocyte replication and markers of cellular senescence. These proteins include $p21^{Cip1}$, $p27^{Kip1}$, $p16^{INK4a}$, $p19^{ARF}$, and p53 (total, bound to p90 and p57-58 isoforms of Mdm2, and free). Optical density (OD) is shown in online data supplement.

Myocyte Size, Oxidative Stress, p53, $p16^{INK4a}$, and Myocyte Death

In the aging failing heart, a subpopulation of myocytes undergoes DNA replication and mitosis, another undergoes hypertrophy, and another cell death.[14] In animals and humans, small myocytes are the only myocytes capable of replicating DNA.[5,14,15] There is an upper limit in myocyte volume, ≈90 000 μm^3, and cross-sectional area, ≈600 to 900 μm^2, beyond which no further hypertrophy occurs.[5,14] They are old cells positive for $p16^{INK4a}$ and p53.[15] The high proficiency of these myocytes to die might be linked to accumulation of oxidative products that exceeds cellular repair mechanisms. The interdependence of oxidative damage and cell death is well established in several systems including myocytes.[15,16] However, the role of cell dimension and age in this process remains to be determined.

Oxidative stress, identified by 8-hydroxy-2′-deoxyguanosine (8-OH-dG), varied from 200 to 1000 pixels (pixels×fluorescence/fluorescence of propidium iodide per nucleus) in apoptotic myocytes with cross-sectional area 400 to 600 μm^2. Higher signals were detected in apoptotic myocytes with cross-sectional area 700 to 900 μm^2 (Figures 2A and 2B). With myocyte necrosis (Figures 2C and 2D), the oxidative challenge was higher ranging from 600 to 5000 pixels. In both forms of death, the intensity of the 8-OH-dG signal correlated directly with myocyte size, $p16^{INK4a}$, p53, and age (Figure 2E).

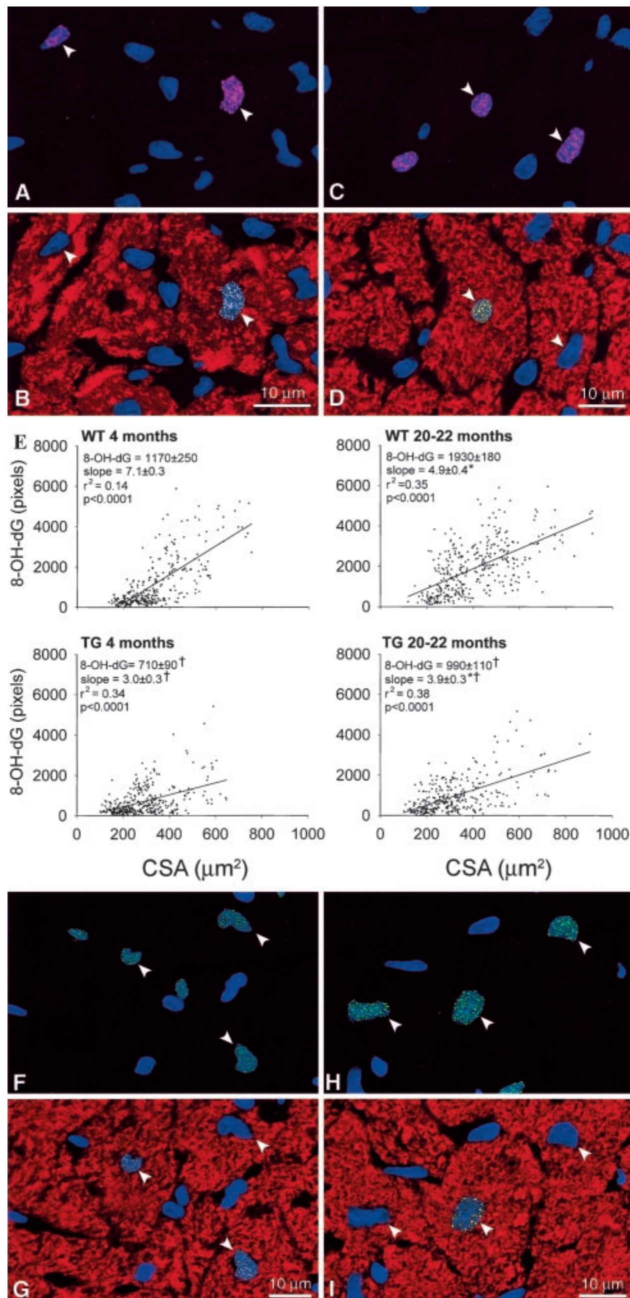

Figure 2. Oxidative stress and myocyte death. WT hearts at 10 to 12 and 20 to 22 months: A, Two nuclei [blue, propidium iodide (PI)] are positive for 8-OH-dG (magenta dots; arrowheads) and one of the two (B) is also labeled by hairpin 1 (white dots, arrowhead). Apoptotic nucleus belongs to a large myocyte (red; α-sarcomeric actin). C and D, Myocyte necrosis: yellow dots (D, arrowhead) correspond to hairpin 2. E, Results are presented as scatter plots and as mean±SD. * and † Indicate statistical difference ($P<0.05$) versus WT and animals at 4 months, respectively. F, Three nuclei express p16^{INK4a} (green dots; arrowheads) and two of the three (G) are labeled by hairpin 1 (white dots, arrowheads). Apoptotic nuclei belong to large myocytes. H and I, Myocyte necrosis: yellow dots (I, arrowheads) correspond to hairpin 2.

Myocyte apoptosis increased 4-fold from 4 (149±60/10^6) to 10 to 12 (622±174/10^6) months and 12-fold from 4 to 20 to 22 (1804±352/10^6) months ($P<0.001$). Corresponding increases in myocyte necrosis were 4-fold (317±70/10^6 versus 1318±352/10^6) and 30-fold (317±70/10^6 versus 9510±2167/10^6) ($P<0.001$). Cell necrosis was 2-fold, 2-fold, and 5-fold greater than apoptosis at 4, 10 to 12, and 20 to 22 months, respectively ($P<0.001$). Dying myocytes were p16^{INK4a} and p53 positive (Figures 2F and 2G), but not all myocytes expressing these proteins were dying: p16^{INK4a}-positive myocytes were 25±6%, 42±14%, and 82±19%, and p53-positive myocytes were 5±4%, 9±6%, and 15±8% in WT mice at 4, 10 to 12, and 20 to 22 months, respectively.

IGF-1 decreased myocyte death. In comparison with WT, myocyte apoptosis in TG was 11% (132±33/10^6; NS), 68% (197±44/10^6), and 78% (396±96/10^6; $P<0.001$) lower at 4, 10 to 12, and 20 to 22 months, respectively. Decreases in myocyte necrosis in TG were 7% (296±50/10^6; NS), 71% (377±87/10^6), and 93% (666±121/10^6; $P<0.001$). Again, cell death correlated directly with 8-OH-dG, cell size, p16^{INK4a}, p53, and age (Figures 2H and 2I). TG mice had lower percentages of p16^{INK4a} (9±5%, 15±7% and 35±13%) and p53 (3±1%, 5±2% and 9±4%) positive myocytes.

Telomere Dysfunction, Senescence, and Death of Myocytes

Alterations in telomeres result in inhibition of cell replication, myocyte senescence, and death.[5,14,15] Telomeres are protected by a complex of proteins including TRF1, TRF2, uncleaved poly(ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK). Telomere-binding proteins have not been yet identified in myocytes; if present, a decrease in their levels would indicate defects in telomere integrity[17,18] of noncycling myocytes. This could provide a link between telomere shortening or uncapping and senescence and death of these non-cycling myocytes. In fact, TRF2 decreased in WT myocytes from 4 to 10 to 12 months becoming undetectable at 20 to 22 months. Cleaved PARP increased at 20 to 22 months, exceeding the uncleaved form. Telomere dysfunction was aggravated by downregulation of the components of DNA-PK with age: heterodimeric complex Ku86/Ku70 and DNA-PK catalytic subunit. TRF1 was not influenced by aging. The impact of aging on telomeric proteins was attenuated in TG myocytes (Figure 3).

Hypophosphorylated RB was predominant in aging WT myocytes reflecting the upregulation of p16^{INK4a}. IGF-1 produced an opposite effect; phosphorylated RB was several-fold higher than hypophosphorylated RB and the phosphorylated-to-hypophosphorylated RB ratio increased with age (Figure 3). This condition may delay myocyte hypertrophy, senescence, and death.

Telomere length is another parameter that regulates cell division and survival in cycling cells[17,18] and might regulate survival of differentiated myocytes. Telomere length in myocyte nuclei, cell cross section, and p16^{INK4a} and p53 expression were plotted as a function of age. There was an inverse relationship between myocyte size and telomere length in WT and TG at 4 and 20 to 22 months of age; larger cells with shorter telomeres were p16^{INK4a} and p53 positive (Figures 4A through 4E; online data supplement). IGF-1 interfered with the age-dependent increases in myocyte size, telomeric shortening and p16^{INK4a} and p53 proteins. In comparison with young cells, telomere length decreased more in old WT than in TG myocytes.

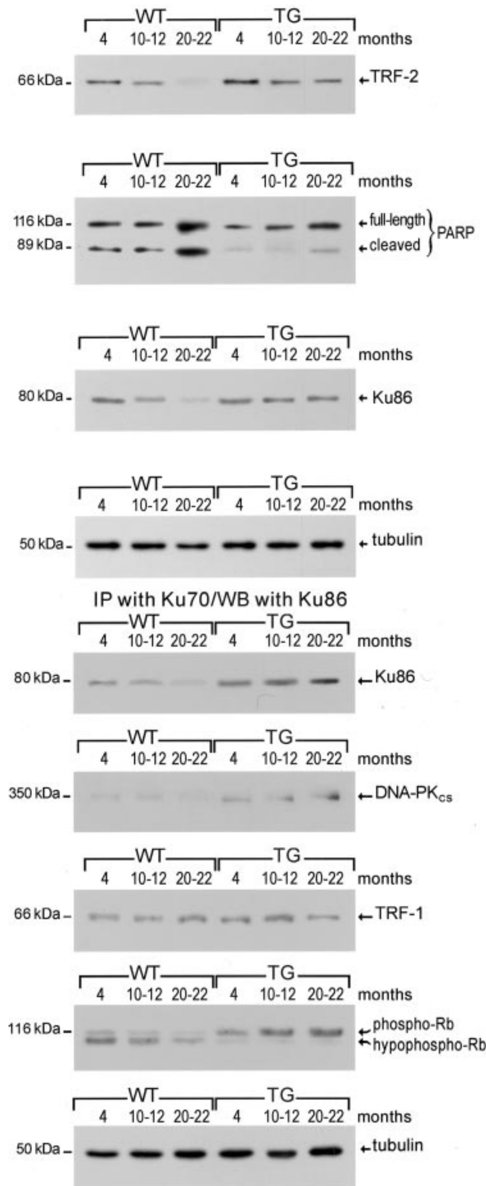

Figure 3. Telomere-related proteins in myocytes. These proteins include TRF-2, PARP, Ku86, Ku70, DNA-PK$_{cs}$, TRF-1, and RB. OD is shown in online data supplement.

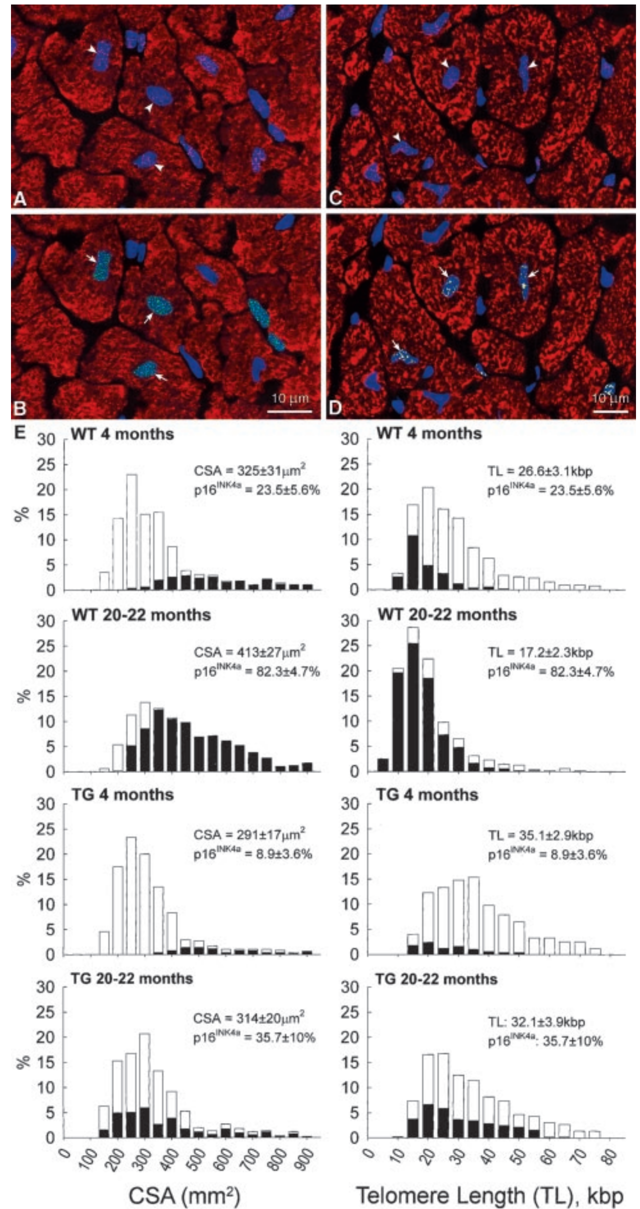

Figure 4. Telomere length and colocalization of p16^{INK4a} and p53 in myocytes. WT hearts at 4 and 20 to 22 months: A, Myocyte (red; α-sarcomeric actin) nuclei (blue, PI) with short telomeres (magenta dots) show low levels of fluorescence (arrowheads). B, Three of these nuclei express p16^{INK4a} (green dots, arrows). A similar finding is demonstrated in C and D. However, yellow dots in D (arrows) correspond to p53. E, Distribution of myocyte cross-sectional areas (CSA) and telomere lengths. Aging in WT results in a shift in the distribution of CSA to the right and telomere lengths to the left. The solid portion of the bars corresponds to p16^{INK4a}-positive myocyte nuclei; they increase with age in both cases. In TG, these various changes are markedly attenuated.

Telomerase Activity and Aging

Telomerase protects chromosomes from telomere erosion, maintains cell replication, and opposes cell death.[19,20] Telomerase competent myocytes are cycling and are p16^{INK4a} and p53 negative. Telomerase identifies young myocytes, actively growing and replacing old dying myocytes.[5,14,19] The telomere-telomerase system is a cell-autonomous determinant of organ aging. The catalytic subunit of telomerase was higher in TG than WT myocytes (Figure 5A). Telomerase activity in WT myocytes decreased from 4 to 20 to 22 months, but increased in TG myocytes. Moreover, enzyme activity was higher in TG than in WT myocytes at each age (Figures 5B through 5D).

IGF-1 activates the PI3K-Akt pathway, and this signaling mechanism may phosphorylate and activate telomerase in TG myocytes. We identified a consensus site for Akt phosphorylation, RVRLRELSQE (amino acids 585 to 594), in the mouse telomerase, suggesting that Akt potentiates telomerase function.[21] Thus, the amino acid sequence was labeled with fluorescein and used in an Akt-kinase assay. Noninfected myocytes and myocytes infected with an adenovirus carrying a nuclear (Akt-nuc) or membrane (Akt-myr) targeted Akt or

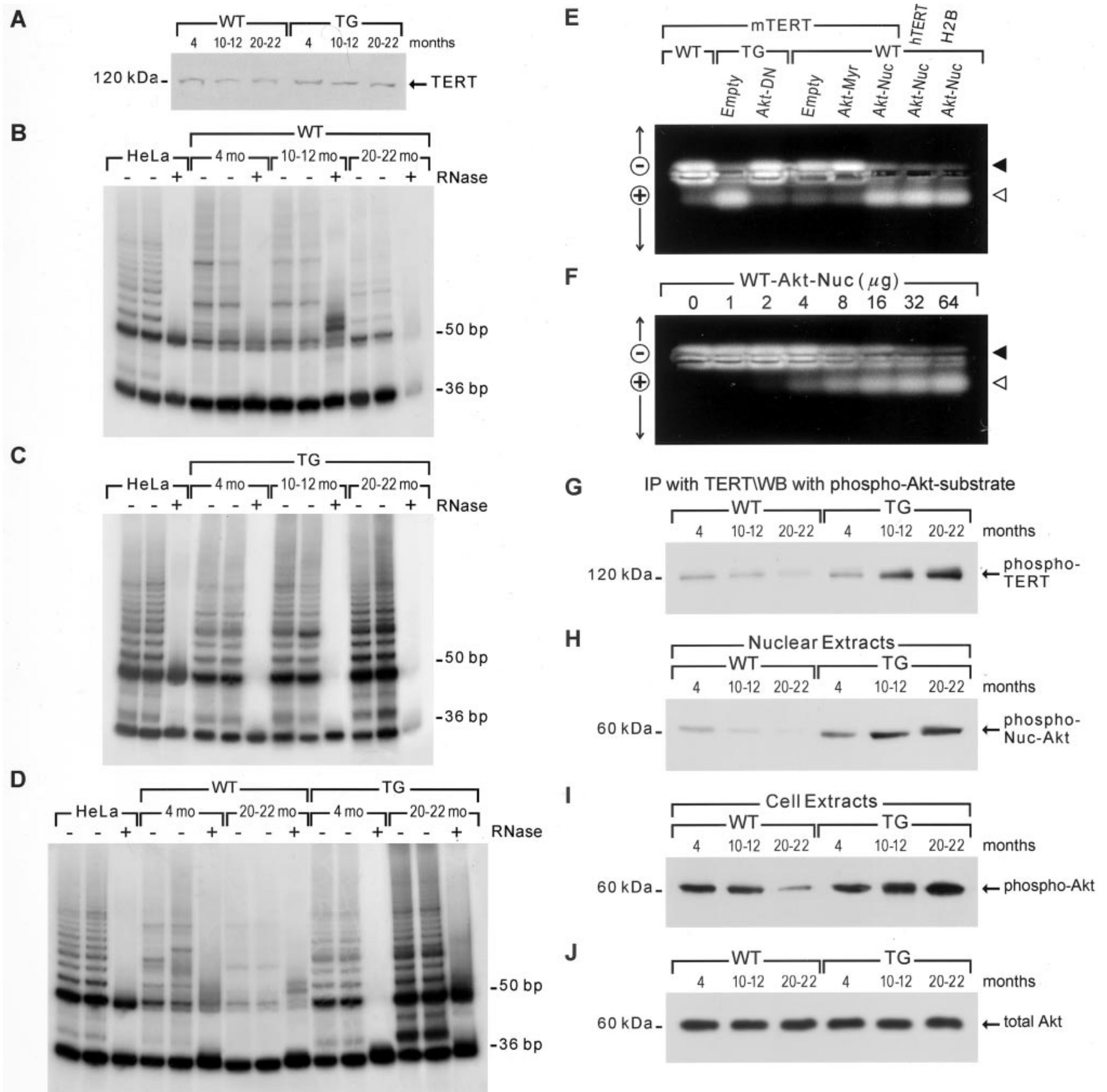

Figure 5. Telomerase and telomerase activity. A, Catalytic subunit of telomerase (TERT) in myocyte nuclear lysates. B through D, Products of telomerase activity start at 50 bp and display a 6-bp periodicity. Myocytes treated with RNase (+) were used as negative control and HeLa cells as positive control. Serial dilution of myocyte proteins (0.5 and 1.0 μg) was used to confirm the specificity of the reaction. E, Akt kinase assay of WT and TG myocyte nuclear lysates and nuclear lysates of WT and TG myocytes infected with empty, Akt-myr, Akt-nuc, or Akt-DN vectors. Substrate utilized in the reaction corresponds to the sequence 585 to 594 of the mouse TERT. Human TERT (hTERT) and histone 2B (H2B) were used as positive controls. F, Increasing amounts of proteins were used to confirm the specificity of the reaction. G through J, Immunoblots of phospho-TERT, phospho-Akt, and total Akt in nuclear and whole-cell lysates. K through M, Telomerase activity in myocytes infected with empty, Akt-myr, Akt-nuc, and Akt-DN vectors. OD is shown in online data supplement.

dominant-negative form of Akt (Akt-DN) were used. Four observations were made (Figures 5E and 5F): (1) At 20 to 22 months, noninfected TG myocytes had higher levels of phosphorylated telomerase sequence than WT myocytes; (2) Akt-nuc infection was involved in phosphorylation of telomerase sequence of WT myocytes; (3) Akt-DN decreased substrate phosphorylation in TG myocytes; and (4) peptide phosphorylation was proportional to Akt-nuc–infected WT myocyte proteins.

Phosphorylation of telomerase by Akt was determined in vivo to identify the role of Akt in telomerase function. Telomerase protein was immunoprecipitated from freshly isolated WT and TG myocytes and was then exposed to an antibody against its phosphorylated Akt consensus site (Fig-

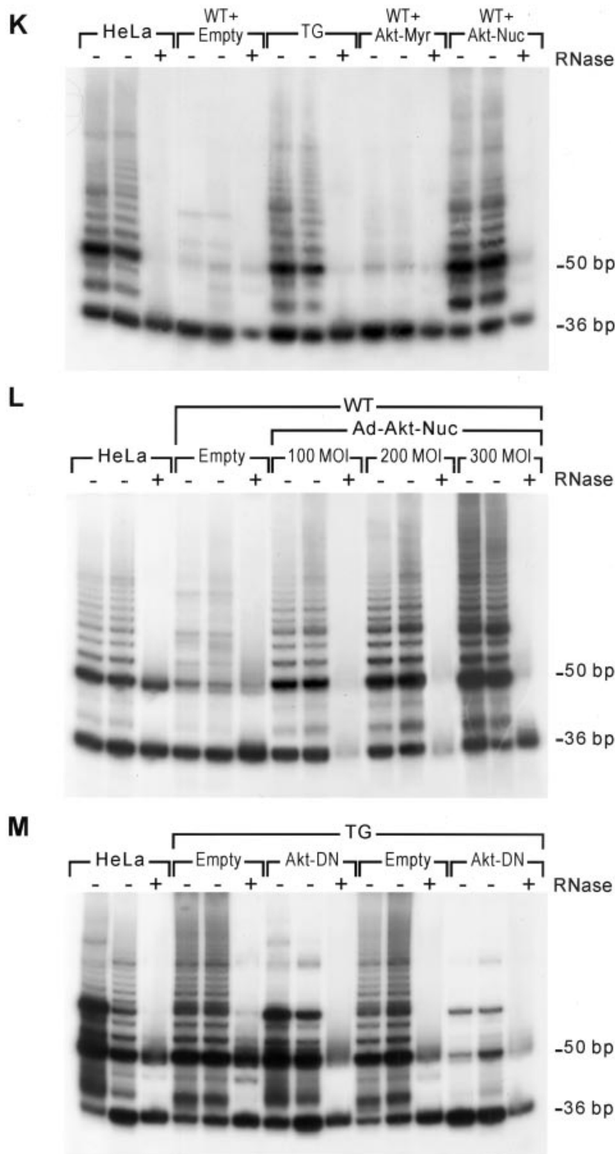

Figure 5 (continued).

ure 5G). Phosphotelomerase level decreased in WT myocytes, with age becoming barely detectable at 20 to 22 months. Conversely, phosphotelomerase increased progressively as a function of age in TG myocytes. At all intervals, phosphotelomerase was higher in TG than in WT myocytes, paralleling the results of telomerase activity described earlier (see Figures 5B through 5D).

The expression of Akt was measured in aging WT and TG myocytes. Akt protein (total) remained constant and did not differ in WT and TG myocytes. However, phospho-Akt levels decreased in WT and increased in TG myocytes from 4 to 20 to 22 months (Figures 5H through 5J). These results are consistent with attenuation and increase in telomerase activity in aging WT and TG myocytes, respectively. To confirm phospho-Akt function in the regulation of telomerase, a TRAP assay was performed in old WT myocytes infected with Akt-nuc or Akt-myr. Only Akt-nuc expression resulted in a marked elevation in telomerase activity. Increasing degrees of Akt-nuc infection of WT myocytes were accompanied by increases in telomerase activity. Akt-myr infection did not modify baseline telomerase in WT myocytes. Telomerase activity in old TG myocytes was severely depressed after infection with the Akt-DN adenovirus (Figures 5K through 5M).

Aging, Myocyte Growth, Number, and Contractile Performance

Myocyte aging in WT mice typically shows alterations in telomeres, telomerase activity, oxidative stress response, and expression of $p16^{INK4a}$ and p53. These intrinsic determinants of cellular senescence promote cell death and impair cell growth, compromising cardiac structure and function. Myocyte loss increases with age more than cell regeneration and the excess in cell dropout leads to ventricular decompensation, suggesting that the aging myopathy is mediated by molecular defects in myocytes. If this were the case, attenuation of cell death and/or potentiation of cell growth should preserve ventricular performance. These conditions are operative in IGF-1 TG mice.

For this purpose, the number of dying myocytes (hairpin 1 and hairpin 2) in the left ventricle was compared with the number of forming cells (BrdU). In WT, the number of dying myocytes was significantly higher than that of replicating cells and this difference increased from 4 to 20 to 22 months. Conversely, in TG, the values of cell death and cell proliferation were comparable at all ages. The rate of myocyte formation was ≈3-fold higher in TG, and the rate of cell death was ≈4-fold higher in WT. Therefore, the total number of ventricular myocytes decreased by 33% in WT at 20 to 22 months, whereas myocyte number remained constant in TG mice (Figure 6). Myocyte loss in aging WT was associated with elevation in LVEDP and a significant decrease in LVDevP and + and − dP/dt (Figure 6). Ventricular dysfunction was absent in TG mice; cell regeneration maintained constant myocyte number in the old heart. The cellular and functional indices of TG hearts at 20 to 22 months were comparable with those of WT hearts at 10 to 12 months.

At all ages, TG myocytes had higher peak shortening, velocity of shortening, and relengthening than WT. Myocyte contractility decreased with age in WT and remained constant in TG, amplifying the difference between old WT and TG. Developed Ca^{2+} was higher in TG than WT myocytes at high rate of stimulation. L-type Ca^{2+} current differed in amplitude being higher in young and old TG than in WT, suggesting that TG myocytes have a more efficient SR with enhanced reuptake and improved diastolic relaxation. WT myocytes manifested a longer recovery from inactivation than TG with age (Figures 7A and 7B; online data supplement). Thus, the mechanical behavior of myocytes contributed to the preservation of ventricular hemodynamics in TG with age.

CSC Aging

Clusters of primitive and early committed cells expressing the stem cell antigen c-kit were found in WT and TG myocardi-

um (Figure 8A). These cells were lineage negative (see expanded Materials and Methods) or expressed the transcription factors GATA-4 and MEF2C, which are implicated in cardiac and myocyte differentiation, respectively.[5,6,14] Moreover, α-sarcomeric actin, cardiac myosin heavy chain, connexin43, and N-cadherin were detected. New small myocytes were telomerase competent and Ki67 positive (Figures 8B and 8C), representing amplifying cells that divided rapidly and differentiated.[5,6,14] The recognition of cells positive for c-kit, GATA-4, and cardiac myosin provided an indisputable link between primitive cells, early differentiating myocytes, and mature parenchymal cells (Figure 8D).

The number of c-kit^{POS} cells in the left ventricle was 47% lower in WT than in TG at 4 months and this difference increased to 56% at 20 to 22 months (Figure 8E). From 4 to 20 to 22 months, there was an increase of 1.9- and 2.3-fold in the number of c-kit^{POS} cells in WT and TG, respectively. However, the expression of p16^{INK4a} was detected in 70% of WT c-kit^{POS} cells, indicating a block in their ability to reenter the cell cycle and divide. This phenomenon involved only 16% of TG c-kit^{POS} cells (Figure 8F). Importantly, apoptosis was 3-fold and 5-fold higher in WT than in TG c-kit^{POS} cells at 4 and 20 to 22 months, respectively. These results on cell death amplified the difference in the pool size of functionally competent CSCs between WT and TG. As in myocytes, all apoptotic c-kit^{POS} cells expressed p16^{INK4a} (Figures 8G and 8H).

To estimate the length of telomeres in c-kit^{POS} cells, a Q-FISH with a probe complementary to the telomere sequence was performed. Nuclei with short telomeres expressed p16^{INK4a} in both WT and TG c-kit^{POS} cells. The combination of short telomeres and p16^{INK4a} was more prominent in WT than in TG c-kit^{POS} cells at 4 and 20 to 22 months. Additionally, the largest fraction of c-kit^{POS} cells labeled by p16^{INK4a} was detected in old WT that had the shortest telomeres (Figure 8I).

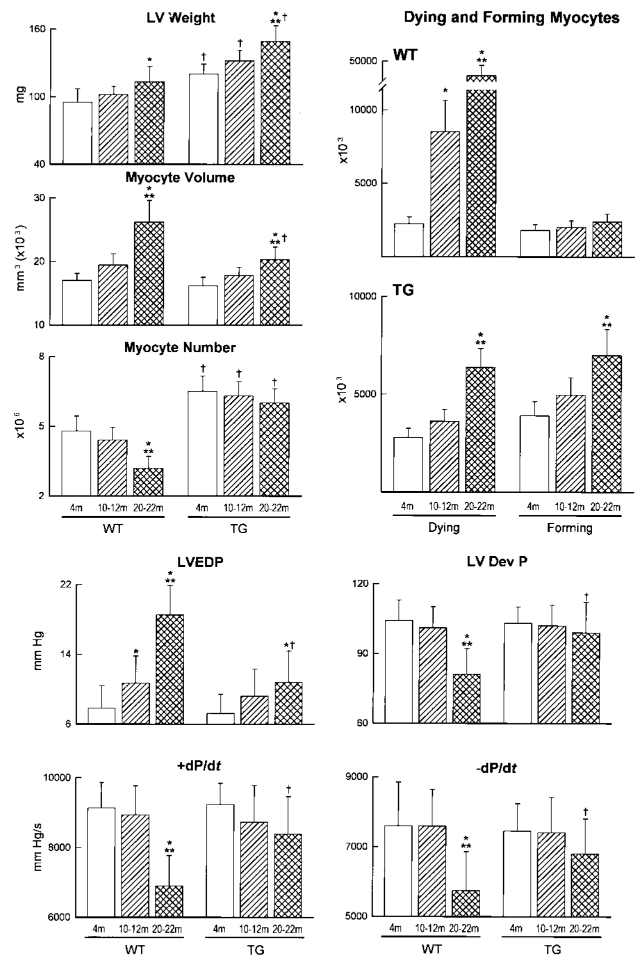

Figure 6. Myocyte volume and number and left ventricular (LV) function. Results are mean±SD. Symbols indicate statistical difference ($P<0.05$) versus animals at 4* and 10 to 12** months and WT† mice. LVEDP indicates LV end-diastolic pressure; LVDevP, LV developed pressure.

Discussion

Decompensated Aging Heart

The objective of this study was to establish whether aging of the heart leads to molecular modifications of myocytes that profoundly alter the characteristics and performance of the old heart resulting in a failing myopathy. These cellular age-dependent effects are dictated by senescence of CSCs, which become largely unable to divide and generate new functionally competent myocytes. CSC apoptosis further attenuates the growth reserve of the myocardium as well as its ability to replace dying myocytes and respond adequately to the increases in physiological loads imposed by myocyte dropout. Myocytes lose with time their youth; the myocyte compartment becomes progressively less heterogeneous with large cells comprising the majority of the myocardium. The normal spectrum of developing, maturing, and senescent myocytes, typical of the adult and middle-aged heart, is no longer apparent and hypertrophied myocytes predominate.

Cardiac aging may be viewed as a limitation in the replication and commitment of CSCs that affects the turnover of myocytes favoring cell loss and the accumulation of old poorly contracting cells. These cells cannot be adequately replaced by new amplifying myocytes undergoing cell division and maturation[5,14] in order to meet the functional needs of the aged heart.[2,22] A similar phenomenon is operative in other organs.[11,18] Cell death coupled with an increased number of myocytes with depressed contractile behavior underlies the onset of cardiac decompensation. The results in WT are consistent with observations in rats[4] and in large animals,[23] although not all studies are in agreement.[24] Heart failure in individuals 66 years of age and older, up to 103 years, has no other confounding variables in nearly 50% of the cases, suggesting that age may be a primary cause of cardiac decompensation and diastolic dysfunction.[25]

In TG mice, CSCs are more numerous and actively growing enhancing myocyte turnover, preserving myocyte number, attenuating the accumulation of hypertrophied myocytes, and increasing the number of new functionally efficient young cells. Together, these factors maintain ventricular function and prevent heart failure, strengthening the contention that cardiac aging occurs at the cellular level. These findings are at variance with the view that IGF-1 promotes premature aging in fruit flies, nematodes, and mice[26] but are consistent with the notion that IGF-1 delays aging and heart failure in humans.[27] However, it is difficult to discriminate

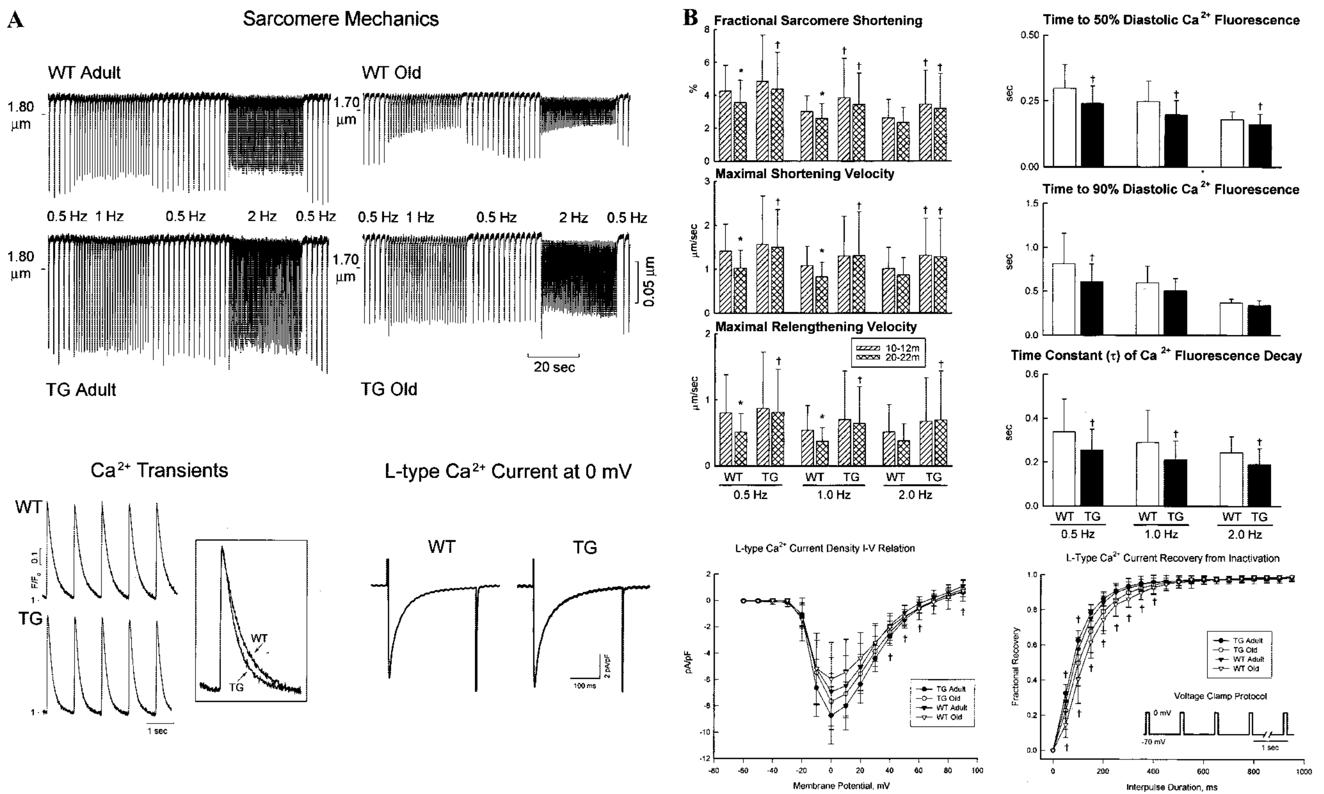

Figure 7. Contractile activity in myocytes. A and B, Representative tracings illustrating peak shortening and velocity of shortening and relengthening, Ca^{2+} transients, and L-type Ca^{2+} current. Results are mean±SD. * and † Indicate statistical difference ($P<0.05$) versus animals at 10 to 12 months and WT mice, respectively.

whether changes in CSCs are primary or secondary events of the aging cardiomyopathy.

Myocyte Senescence

Cellular senescence is characterized by biochemical events that occur within the cell leading to growth arrest and loss of specialized cellular functions.[11,18] These defects, such as repeated oxidative stress, may evolve into the activation of cell death or result in the development of a senescent phenotype. The old cells in the heart do not differ from those in other organs.[28] Senescent cells cannot divide, their ability to synthesize proteins or secrete autacoids and hormones is reduced, and the antioxidant defense mechanisms and the DNA repair system are attenuated.[29] In myocytes, in which cell replication is already lost, aging leads to a block in the capacity to hypertrophy.[5,13] Myocyte mechanics and Ca^{2+} transients are altered indicating that the systems regulating activation, contraction and relaxation are defective. A preservation of myocyte mechanical behavior coupled with a reduced response to norepinephrine has been seen in aging Wistar rats.[30,31] Importantly, there is an upper limit to myocyte growth; cells with cross-sectional areas 300 to 500 μm^2 do not replicate but can hypertrophy, whereas myocytes 600 to 900 μm^2 do not incorporate BrdU, do not hypertrophy and express $p16^{INK4a}$ and/or p53.[5,14,15]

p53 modulates apoptosis and senescence by increasing the expression of specific proteins, including Bax, Bad, and $p21^{Cip1}$. Bax and Bad are implicated in apoptosis, whereas high levels of $p21^{Cip1}$ are involved in irreversible growth arrest and cellular senescence.[18] Low quantities of $p21^{Cip1}$, however, result in transient inhibition of cell growth. $p16^{INK4a}$ blocks the cell cycle by maintaining RB in its hypophosphorylated state; RB interferes with the cell cycle by repressing the E2F transcription factors. Therefore, $p16^{INK4a}$ is an ideal inducer of cellular senescence. The $p16^{INK4a}$/RB pathway promotes also apoptosis by increasing p53 through Mdm2 downregulation.[13,32] Why a cell undergoes apoptosis or senescence in response to identical stimuli is unclear. However, apoptosis and senescence are influenced by the intensity and duration of the inciting stimulus, the past history of the cell, and environmental cues.[18] IGF-1 in myocytes counteracts these variables, limiting the expression of age-related genes and cell death inducers.

Myocytes, Telomeres, and Telomerase

Chromosomal ends contain long stretches of hexameric sequences, the telomeres, which bind specific proteins. Telomerase and telomere-binding proteins maintain telomere integrity and cell viability.[15,19,20] The presence of short or uncapped telomeres discriminates old from young cells and conditions cell fate.[19] In WT, enlarged cells with shortened and uncapped telomeres characterize myocyte senescence. Telomere attrition initiating the senescence program or cell death is identical in young and old myocytes but the number of cells involved differs in magnitude. Senescent WT myo-

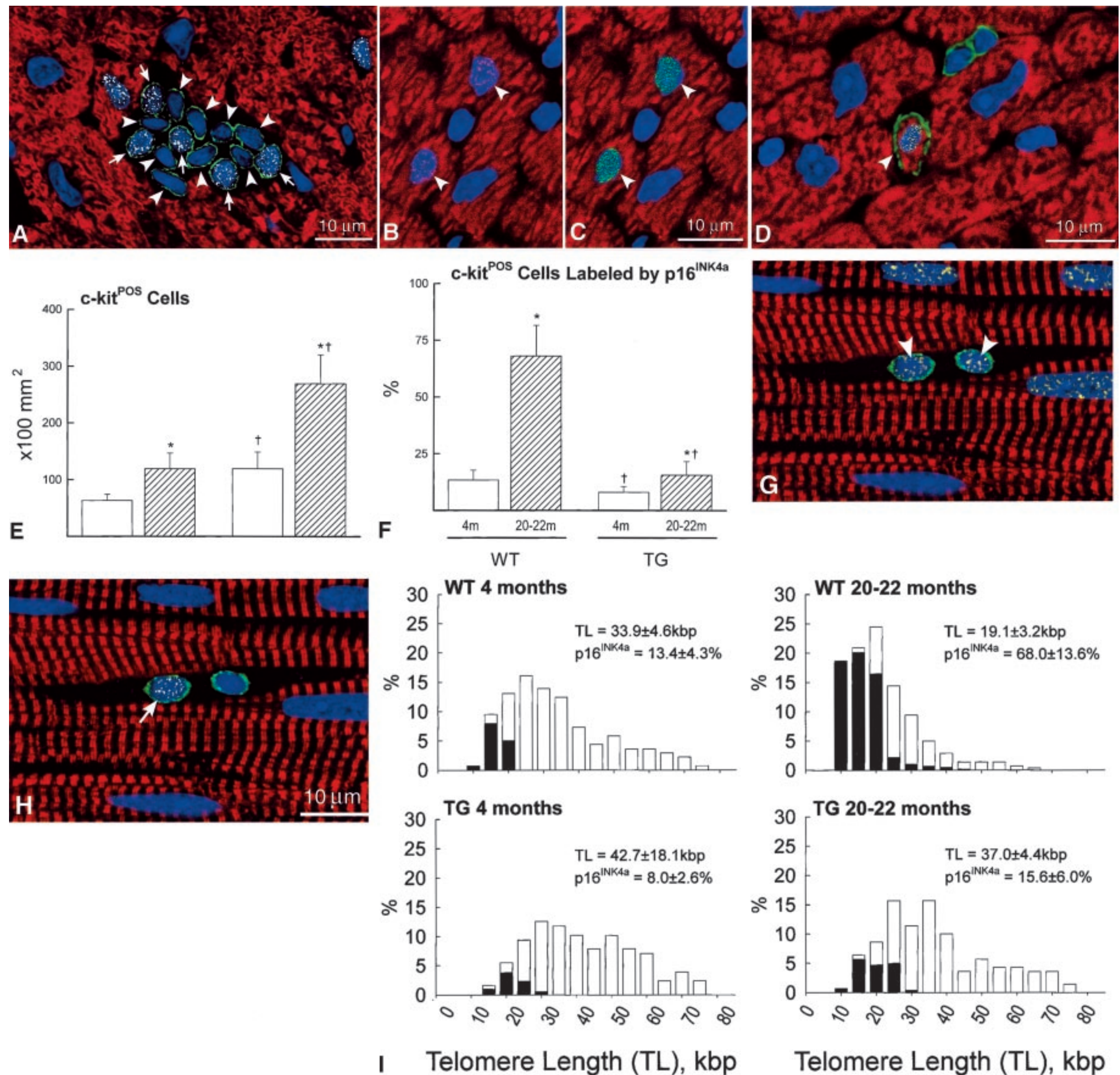

Figure 8. Cardiac stem cells. WT heart at 4 months: A, cluster of c-kit–positive cells (green, arrowheads). Five of these cells express GATA-4 (white dots, arrows) in nuclei (blue, PI). Myocytes (red; α-sarcomeric actin). B and C illustrate the same field. Magenta fluorescence dots in B correspond to telomerase (arrowheads) in two myocyte nuclei. Green dots in C depict Ki67 labeling in the same two nuclei (arrowheads). D, One c-kit–positive cell (green, arrowhead) expresses MEF2C (yellow) in the nucleus and α-sarcomeric actin in the cytoplasm (red). E and F, Results are mean$\pm$SD. * and † Indicate statistical difference ($P<0.05$) versus animals at 4 months and WT mice, respectively. G and H illustrate the same field in a WT heart at 20 to 22 months. Two c-kit–positive cells (green) express $p16^{INK4a}$ (yellow dots, arrowheads) in their nuclei (blue, PI). One c-kit-$p16^{INK4a}$–positive cell is labeled by hairpin 1 (white dots, arrow). I, Distribution of telomere length in CSCs. Aging in WT results in a shift to the left in the distribution of telomere lengths. Solid bars correspond to $p16^{INK4a}$-positive CSC nuclei; they increase with age. In TG, these changes are markedly attenuated.

cytes have telomeres of 15 kbp instead of 30 kbp, express $p16^{INK4a}$, p53, and show modest levels of 8-OH-dG in the nucleus. However, cell death is coupled with high levels of 8-OH-dG. IGF-1 overexpression does not change the properties of senescent and dying myocytes in terms of telomere length and expression of $p16^{INK4a}$ and p53. IGF-1 reduces myocyte senescence and death; telomerase activity is enhanced in younger myocytes and this enzyme protects from telomeric shortening, oxidative injury, growth arrest, and cell death. The expression of proteins implicated in DNA repair and telomere integrity, Ku86/Ku70, PARP, and TRF2, decreases with age in WT but remains constant in TG, pointing to an antiaging effect of IGF-1. Telomere erosion at each round of cell division, oxidative stress, and downregulation of telomeric proteins define myocyte senescence.

Cardiac Stem Cells

The process of CSC senescence follows a pattern similar to that identified in this study in myocytes of rodents and previously in humans.[14] This process is characterized by the expression of $p16^{INK4a}$ and telomeric shortening. Because of this CDK inhibitor, a large number of c-kit^{POS} cells cannot divide symmetrically or asymmetrically and maintain the CSC pool. The increase in c-kit^{POS} cells undergoing senes-

cence and death interferes with the needs for myocyte replacement in the old heart. This deficiency promotes differentiation of the remaining p16^{INK4a}-negative c-kit^{POS} cells into amplifying myocytes. The higher fraction of CSCs undergoing myocyte commitment is consistent with the increase in BrdU labeling of WT myocytes with age. CSC senescence, on the one hand, and differentiation, on the other, leads to a progressive depletion of the growth reserve of the heart. Although transgene expression was myocyte restricted, overexpression of the secreted form of IGF-1 had an impact on CSC function. IGF-1 accumulates at high concentration in myocytes where it is then secreted in the interstitium.[9] The released IGF-1 binds to IGF-1 receptors present on CSCs (unpublished data, 2004). The IGF-1/IGF-1 receptor system induces CSC division, enhances telomerase activity, delays senescence, and preserves the reservoir of functionally competent CSCs (unpublished data, 2004). But whether IGF-1–mediated attenuation of p53, angiotensin II formation, oxidative stress, and myocyte death[14,16,32] is operative in CSCs remains to be determined. Importantly, IGF-1 suppresses the ASK-1–induced activation of JNK/p38 kinase and apoptosis.[33] In fact, the expression of IGF-1 increases in the surviving myocytes after infarction and this response prevents the activation of myocyte apoptosis in the overloaded spared myocardium positively interfering with side-side slippage, ventricular dilation, and wall thinning of the postinfarcted heart.[34,35] Thus, CSCs and IGF-1 protect myocardial regeneration and delay organ aging and heart dysfunction.

Akt and Myocardial Aging

On the basis of this discussion, cardiac aging is a time-dependent process that consistently attenuates myocyte turnover mediated by growth limitation at the level of the controlling cell, the CSC. In the old heart, the myocyte commitment of CSCs is severely reduced by senescence and death of this cell compartment and the postulated decrease in the number of rounds of division of amplifying myocytes. Telomerase activity is markedly decreased in amplifying myocytes leading to premature telomeric shortening and growth arrest. The principal defect seems to be linked to diminished nuclear expression of phospho-Akt. This kinase has the ability to promote phosphorylation of telomerase enhancing enzyme activity and, thereby, cell growth and survival delaying the onset of the aging myopathy. The PI3K-Akt signaling pathway is partially rescued by IGF-1 overexpression that upregulates nuclear phospho-Akt function and telomerase.

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References

1. Chien KR, Olsen EN. Converging pathways and principles in heart development and disease. *Cell*. 2002;110:153–162.
2. Lakatta EG. Cardiovascular regulatory mechanisms in advanced age. *Physiol Rev*. 1993;73:413–467.
3. Troen BR. The biology of aging. *Mt Sinai J Med*. 2003;70:3–22.
4. Anversa P, Nadal-Ginard B. Myocyte renewal and ventricular remodeling. *Nature*. 2002;415:240–243.
5. Urbanek K, Quaini F, Tasca G, Torella D, Castaldo C, Nadal-Ginard B, Leri A, Kajstura J, Quaini E, Anversa P. Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. *Proc Natl Acad Sci U S A*. 2003;100:10440–10445.
6. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti C, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell*. 2003;114:763–776.
7. Johnson FB, Sinclair DA, Guarente L. Molecular biology of aging. *Cell*. 1999;96:291–302.
8. Winn N, Paul A, Musaro A, Rosenthal N. Insulin-like growth factor isoforms in skeletal muscle aging, regeneration, and disease. *Cold Spring Harb Symp Quant Biol*. 2002;67:507–518.
9. Reiss K, Cheng W, Ferber A, Kajstura J, Li P, Li B, Olivetti G, Homcy CJ, Baserga R, Anversa P. Overexpression of insulin-like growth factor-1 in the heart is coupled with myocyte proliferation in transgenic mice. *Proc Natl Acad Sci U S A*. 1996;93:8630–8635.
10. Stein GH, Drullinger LF, Soulard A, Dulic V. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol Cell Biol*. 1999;19:2109–2117.
11. Beausejour CM, Krotolica A, Galimi F, Narita M, Lowe SW, Yaswen P, Campisi J. Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J*. 2003;22:4212–4222.
12. Gartel A, Tyner A. Transcriptional regulation of the p21$^{(WAF1/CIP1)}$ gene. *Exp Cell Res*. 1999;246:280–289.
13. Weber JD, Kup ML, Bothner B, DiGiammarino EL, Kriwacki RW, Roussel MF, Sherr CJ. Cooperative signals governing ARF-mdm2 interaction and nucleolar localization of the complex. *Mol Cell Biol*. 2000;20:2517–2128.
14. Chimenti C, Kajstura J, Torella D, Urbanek K, Heleniak H, Colussi C, Di Meglio F, Nadal-Ginard B, Frustaci A, Leri A, Maseri A, Anversa P. Senescence and death of primitive cells and myocytes leads to premature cardiac aging and heart failure. *Circ Res*. 2003;93:604–613.
15. Leri A, Franco S, Zacheo A, Barlucchi L, Chimenti S, Limana F, Nadal-Ginard B, Kajstura J, Anversa P, Blasco MA. Ablation of telomerase and telomere loss leads to cardiac dilation and heart failure associated with p53 upregulation. *EMBO J*. 2003;22:131–139.
16. Frustaci A, Kajstura J, Chimenti C, Jakoniuk I, Leri A, Maseri A, Nadal-Ginard B, Anversa P. Myocardial cell death in human diabetes. *Circ Res*. 2000;87:1123–1132.
17. Goytisolo FA, Blasco MA. Many ways to telomere dysfunction: in vivo studies using mouse models. *Oncogene*. 2002;21:584–591.
18. Campisi J. Cellular senescence and apoptosis: how cellular responses might influence aging phenotypes. *Exp Gerontol*. 2002;38:5–11.
19. Blasco MA. Telomerase beyond telomeres. *Nat Rev Cancer*. 2002;2:627–633.
20. Leri A, Barlucchi L, Limana F, Deptala A, Darzynkiewicz Z, Hintze TH, Kajstura J, Nadal-Ginard B, Anversa P. Telomerase expression and activity are coupled with myocyte proliferation and preservation of telomeric length in the failing heart. *Proc Natl Acad Sci U S A*. 2001;98:8626–8631.
21. Kang SS, Kwon T, Kwon DY, Do SI. Akt protein kinase enhances human Telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J Biol Chem*. 1999;274:13085–13090.
22. Wei JY. Age and the cardiovascular system. *N Engl J Med*. 1992;237:1735–1739.
23. Vaitkevicius PV, Lane M, Spurgeon H, Ingram DK, Roth GS, Egan JJ, Vasan S, Wagle DR, Ulrich P, Brines M. A cross-link breaker has sustained effects on arterial and ventricular properties in older rhesus monkeys. *Proc Natl Acad Sci U S A*. 2001;98:1171–1175.
24. Takagi G, Asai K, Vatner SF, Kudej RK, Rossi F, Peppas A, Takagi I, Resuello RR, Natividad F, Shen YT, Vatner DE. Gender differences on the effects of aging on cardiac and peripheral adrenergic stimulation in old conscious monkeys. *Am J Physiol*. 2003;285:H527–H534.
25. Kitzman DW. Diastolic heart failure in the elderly. *Heart Fail Rev*. 2002;7:17–27.
26. Lithgow GJ, Gill MS. Cost-free longevity in mice? *Nature*. 2003;421:125–126.
27. Vasan RS, Sullivan LM, D'Agostino RB, Roubenoff R, Harris T, Sawyer DB, Levy D, Wilson PWF. Serum insulin-like growth factor I and risk for heart failure in elderly individuals without previous myocardial infarction: the Framingham heart study. *Ann Intern Med*. 2003;139:642–648.

28. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Campisi J. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A*. 1995;92:9363–9367.
29. Hasty P, Campisi J, Hoeijmakers J, van Steeg H, Vijg J. Aging and genome maintenance: lessons from the mouse? *Science*. 2003;299: 1355–1339.
30. Fraticelli A, Josephson R, Danziger RS, Lakatta EG, Spurgeon HA. Morphological and contractile characteristics of rat cardiac myocytes from maturation to senescence. *Am J Physiol*. 1989;257:H259–H265.
31. Sakai M, Danziger RS, Xiao RP, Spurgeon HA, Lakatta EG. Contractile response of individual cardiac myocytes to norepinephrine declines with senescence. *Am J Physiol*. 1992;262:H184–H189.
32. Leri A, Liu Y, Wang X, Kajstura J, Malhotra A, Meggs LG, Anversa P. Overexpression of insulin-like growth factor-1 attenuates the myocyte renin-angiotensin system in transgenic mice. *Circ Res*. 1999; 84:752–762.
33. Galvan V, Logvinova A, Sperandio S, Ichijo H, Bredesen DE. Type 1 insulin-like growth factor receptor (IGF-1R) signaling inhibits apoptosis signal-regulating kinase 1 (ASK1). *J Biol Chem*. 2003;278: 13325–13332.
34. Cheng W, Reiss K, Li P, Chun MJ, Kajstura J, Olivetti G, Anversa P. Aging does not affect the activation of the myocyte insulin-like growth factor-1 autocrine system after infarction and ventricular failure in Fischer 344 rats. *Circ Res*. 1996;78:536–546.
35. Li Q, Li B, Wang X, Leri A, Jana KP, Liu Y, Kajstura J, Baserga R, Anversa P. Overexpression of insulin-like growth factor-1 in mice protects from myocyte death after infarction, attenuating ventricular dilation, wall stress, and cardiac hypertrophy. *J Clin Invest*. 1997;100:1991–1999.