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Telomere-independent cellular senescence in human fetal cardiomyocytes

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Summary

Fetal cardiomyocytes have been proposed as a potential source of cell-based therapy for heart failure. This study examined cellular senescence in cultured human fetal ventricular cardiomyocytes (HFCs). HFCs were isolated and identified by immunocytochemistry and RT-PCR. Cells were found to senesce after 20–25 population doublings, as determined by growth arrest, morphological changes and senescence-associated β-galactosidase activity. Using the telomeric repeat amplification protocol assay, telomerase activity was undetectable in primary HFCs. Cells were transduced to express the human reverse transcriptase subunit (hTERT) of telomerase. This resulted in greatly increased telomerase activity, but no significant lifespan extension. Analysis of telomere length in primary HFCs revealed that the senescent phenotype was not accompanied by telomere shortening. Telomeres in hTERT-positive cells were elongated in comparison with primary cells, and elongation was retained in senescent cells. Levels of the tumor suppressor protein $p16^{INK4A}$ increased in all senescent cells whether telomerase-positive or -negative. Senescence was accompanied by a decline in transcript levels of the polycomb gene Bmi-1, Ets1 and Ets2 transcription factors, and Id1, Id2 and Id3 helix–loop–helix proteins, suggesting roles for these genes in maintenance of cardiomyocyte proliferative capacity. In addition to offering novel insights into the behavior of human fetal cardiomyocytes in culture, these findings have implications for the development of a cell-based therapy for cardiac injury using primary fetal heart tissue.

Key words: cellular senescence; human fetal cardiomyocytes; p16; replicative lifespan; telomerase; telomeres.

Introduction

The regenerative capacity of the adult human heart is extremely limited. Consequently, when heart muscle is damaged and cardiomyocytes are lost, contractile tissue is not significantly regenerated, and scar formation occurs. Cardiac transplantation remains the treatment of choice for end-stage heart failure, but is limited by a shortage of donor organs, complications of immunosuppression and graft vasculopathy (Hunt, 1998). A cell-based therapy for heart failure is extremely attractive as an alternative to cardiac transplantation but has been limited by the lack of a supply of appropriate cells. Unfortunately, adult mammalian cardiomyocytes are predominantly post-mitotic and so cannot be expanded *in vitro*. Therefore, alternative cell sources have been investigated in recent years, with varying and occasionally disputed results. Candidates include fetal cardiomyocytes (Soonpaa *et al*., 1994; Reffelmann *et al*., 2003), skeletal myoblasts (Taylor *et al*., 1998; Reinecke *et al*., 2004) and embryonic stem cells (Dowell *et al*., 2003; Kehat & Gepstein, 2003). Hematopoietic stem cells have been reported to regenerate infarcted myocardium (Orlic *et al*., 2001), although these findings have been called into question (Balsam *et al*., 2004; Murry *et al*., 2004). Recently, progenitor cells in adult heart that show stem cell characteristics have been described (Beltrami *et al*., 2003; Oh *et al*., 2003), and these may provide novel opportunities for myocardial repair.

Unlike the adult, fetal cardiomyocytes *in vitro* have been shown to proliferate (Goldman & Wurzel, 1992; Burton *et al*., 1999). Moreover, transplant studies have shown that fetal cardiomyocytes can survive and become stably integrated into normal and injured adult hearts, leading to beneficial effects upon cardiac function. They are capable of forming intercalated disks with host cardiomyocytes, developing sarcomeric structures and other morphological features of differentiation and becoming functionally coupled (Reffelmann *et al*., 2003; Dowell *et al*., 2003; Rubart *et al*., 2003). These findings suggest that fetal cardiomyocytes may represent the optimal cell type for therapy of heart failure (Chien, 2004).

A major difficulty with utilizing fetal cardiomyocytes is the limited availability of human fetal tissue (Reffelmann *et al*., 2003). Thus, the fact that fetal cardiomyocytes can replicate *in vitro* will be critical to generating a sufficient number of cells for transplantation. Although earlier studies have shown that fetal human cardiomyocytes are capable of proliferating in tissue culture in the presence of serum, those studies did not examine the proliferative capacity in detail (Goldman & Wurzel, 1992; Burton *et al*., 1999). Here, we examine more extensively the proliferative ability and behavior of isolated human fetal cardiomyocytes in culture. We found that cells isolated from 22- to 24-week-gestation human hearts and positively identified as cardiomyocytes have a proliferative capacity of approximately 20 population doublings (PDs) under normal culture conditions. The growth arrest that occurred after 20 PDs had some characteristics of cellular senescence but is not due to telomere shortening as shown by telomere length determination and lack of

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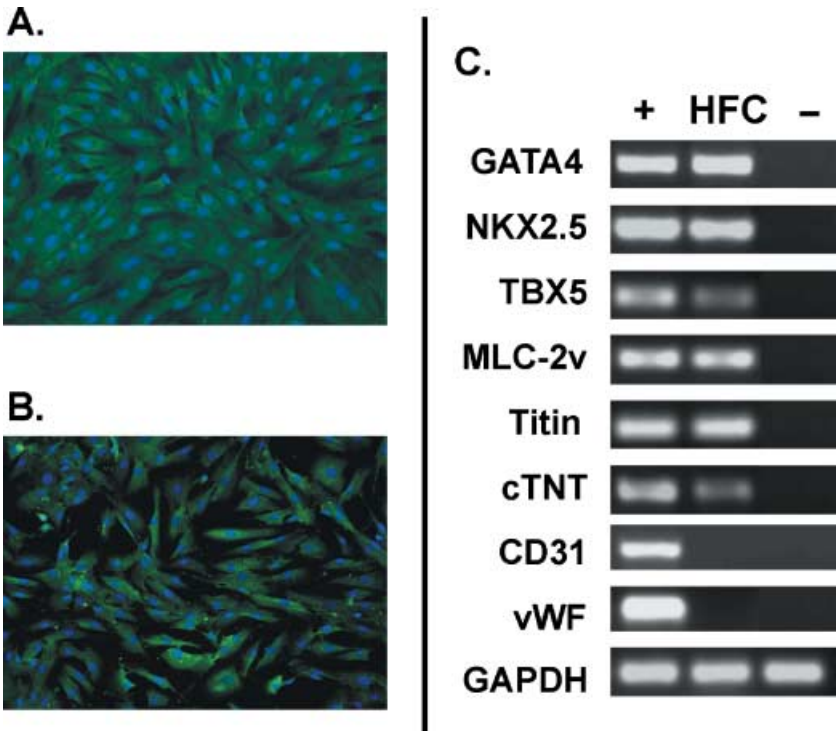

Fig. 1 Identification of proliferating human fetal heart cells (HFC) as cardiomyocytes. (A) Immunostaining of proliferating cells (2 PD) isolated from fetal human heart with monoclonal antibody recognizing sarcomeric myosin. (B) Immunostaining of proliferating cells (2 PD) isolated from fetal human heart with monoclonal antibody recognizing sarcomeric α-actinin. (C) RT-PCR analysis of cardiomyocyte markers in HFC cells. Proliferating cells isolated from fetal human heart were analysed for expression of markers specific for cardiomyocyte lineage and function: GATA4, Nkx2.5, TBX5, myosin light chain-2v, titin and cardiac troponin T. Expression of CD31 and von Willebrand Factor (vWF) was examined to exclude endothelial contamination. Positive controls used were fetal human heart (GATA4, Nkx2.5, TBX5, MLC-2v, Titin, cTNT) and lung (CD31 and vWF). Negative control for each was human diploid fibroblast cDNA. GAPDH was used as a loading control; the band shown in positive control lane is fetal human heart; level of lung GAPDH was equivalent (data not shown).

responsiveness to human reverse transcriptase subunit (hTERT)-induced telomere lengthening.

Results

Identification of isolated cells as cardiomyocytes

The cardiomyocyte isolation procedure of collagenase digestion and gradient centrifugation yielded a population of cells that exhibited a remarkable degree of morphological homogeneity. No spindle-shaped fibroblastic cells could be seen. Immunocytochemical analysis revealed that all cells in the isolated cultures were positive for sarcomeric myosin heavy chain (Fig. 1A) and for α-sarcomeric actinin, a prototypical Z-line protein (Fig. 1B). These results indicate that the initial cultures consisted essentially of pure cardiomyocytes. Negative controls minus primary antibody confirmed that these observations were not due to cellular autofluorescence (data not shown).

To characterize further the cells isolated from the fetal heart, RT-PCR analysis was employed. Figure 1(C) shows mRNA transcript expression of a wide range of markers of cardiomyocyte lineage and function. The isolated fetal heart cells (middle lane) express transcription factors known to regulate cardiomyocyte development – GATA4, Nkx2.5 and TBX5. Other important cardiac genes present were ventricular myosin light chain (MLC-2v), titin and cardiac troponin T (cTNT). Additionally, human fetal ventricular cardiomyocytes (HFCs) did not express CD31 or von Willebrand Factor (vWF), indicating that cultures were not contaminated with endothelial cells. Human diploid fibroblasts were used as negative controls (right lane), and GAPDH served as an internal loading control. Positive controls (left lane) were fetal human heart (GATA4, Nkx2.5, TBX5, MLC-2v, Titin, cTNT, GAPDH) and lung (CD31 and vWF).

Using this semi-quantitative method, transcript levels of all cardiomyocyte markers studied in early passage proliferating isolated heart cells appear to be similar to those in fetal human heart.

Senescence of primary fetal cardiomyocytes in culture

Isolated fetal cardiomyocytes were cultured in Dulbecco's modified Eagle medium with high glucose and 10% fetal bovine serum and were found to proliferate under these conditions. The lifespan of the cells was determined by continuous passage. Cells were found to show steadily increasing doubling times and finally underwent growth arrest after 20–25 PDs (Fig. 2). Growth arrest of primary cells was accompanied by up-regulation of senescence-associated (SA) β-galactosidase activity (Fig. 3C) and increased expression of the $p16^{INK4A}$ tumor suppressor protein (see Fig. 5, lane 4). SA β-galactosidase and $p16^{INK4A}$ are widely recognized as indicators of cellular senescence (Dimri *et al.*, 1995; Sharpless & DePinho, 2004).

Telomerase activity in hTERT-infected and uninfected cells

Telomerase is a multisubunit riboprotein that maintains telomere length in germ cells, stem cells and many immortal cell lines. Telomerase activity is absent in most normal somatic cells due to the lack of expression of the telomerase reverse transcriptase subunit, but can be induced by exogenous expression of that

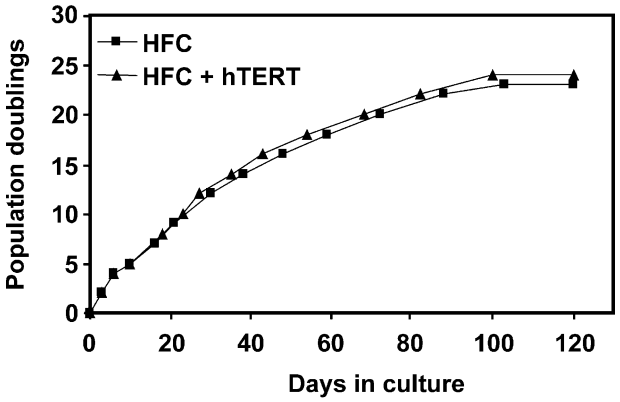

Fig. 2 *In vitro* growth of human fetal cardiomyocyte populations. Number of PDs are plotted as a function of time in culture. Squares represent primary human fetal cardiomyocytes (HFC); triangles represent human fetal cardiomyocytes transduced with hTERT.

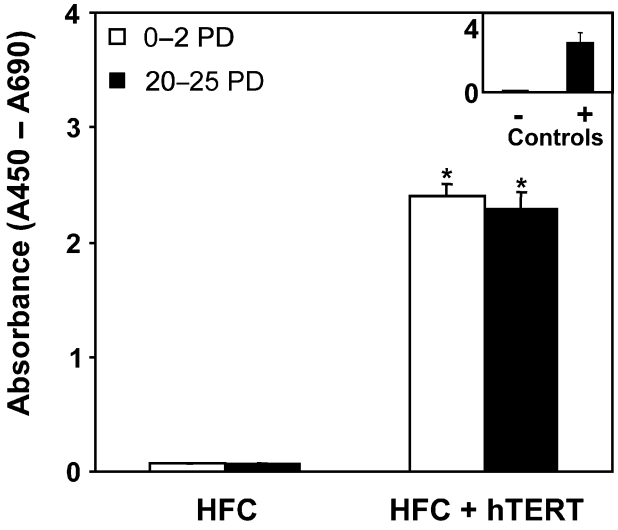

Fig. 4 ELISA-based TRAP assay demonstrating telomerase activity in proliferating and growth-arrested HFC cells with and without the hTERT gene. Inset: positive and negative controls, telomerase-expressing 293 cells and heat-inactivated samples, respectively. Values shown represent mean of three independent assays ± SEM. $*P < 0.001$.

gene, which functions in a species-specific manner. The human homologue, hTERT, has been shown to immortalize primary human fibroblasts (Bodnar *et al*., 1998), which otherwise have a finite lifespan in culture due to cellular senescence. Like fibroblasts, cardiomyocytes are of mesenchymal lineage, and thus we examined whether hTERT could similarly extend the lifespan of primary HFCs in culture. A Moloney murine-derived retroviral vector, LTRTNLlox, was used to introduce hTERT into isolated HFCs. This vector has previously been demonstrated to be capable of extending the proliferative lifespan of primary human fibroblasts and immortalizing human pancreatic cell lines (Halvorsen *et al*., 1999).

Proliferating HFC cells were infected with hTERT expressing retrovirus after approximately 1 PD. hTERT-positive cells were then selected using hygromycin as a selectable marker. Figure 4 shows the relative telomerase activity in the primary HFC cells and hTERT-positive HFC cells. Infection with hTERT significantly ($P < 0.001$) increased telomerase activity in the proliferating cells (0–4 PDs), and its activity was unchanged in both telomerase-positive and -negative cells that had undergone growth arrest (20–25 PDs).

Accumulation of senescence-associated β-galactosidase is independent of telomerase activity

HFC cells, with or without telomerase, were cultured continuously and their proliferative ability determined. In every case, cultured cells entered a period of enlarged morphology and slowed growth. After 20–25 PDs, all cultures were completely growth-arrested (Fig. 2). Loss of proliferation was invariably accompanied by morphological changes, including enlargement and vacuolization of the cell body (Fig. 3C,D). We consistently observed no significant extension of the proliferative lifespan in hTERT-transduced

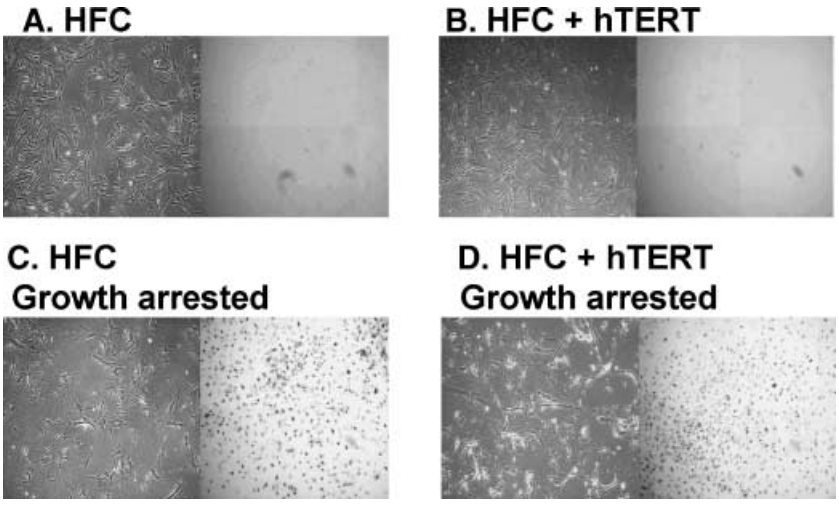

Fig. 3 Phase contrast images of morphological changes and localization of SA β-galactosidase activity over time. (A) Proliferating (6 PD) HFC cells; (B) proliferating (6 PD) telomerase-positive HFC cells; (C) growth-arrested (22 PD) HFC cells; (D) growth-arrested (22 PD) telomerase-positive HFC cells.

cells. Up-regulation of SA β-galactosidase activity has been recognized as a marker of cellular senescence (Dimri *et al*., 1995). SA β-galactosidase expression was detected in growth-arrested primary HFC cells and hygromicin-selected hTERT-positive cells (Fig. 3C,D). The growth arrest was not due to failure of the vector to express hTERT because telomerase activity was present in hTERT-transgenic cells even after the cells stopped dividing, as demonstrated by telomeric repeat amplification protocol (TRAP) (Fig. 4). In proliferating cardiomyocytes, with or without telomerase, activity of SA β-galactosidase was negligible (Fig. 3A,B).

p16^{INK4A} levels in cells undergoing growth arrest

Expression of the p16^{INK4A} tumor suppressor protein increases in senescent cells (Sharpless & DePinho, 2004), and may play a role in senescence-related growth arrest. Therefore, we compared p16^{INK4A} levels in early-passage and growth-arrested cells by Western blot. Extracts prepared from proliferating cardiomyocytes with or without telomerase that had undergone 0–4 PDs contained low levels of p16^{INK4A} protein (Fig. 5, lanes 3 and 5). In sharp contrast, p16^{INK4A} was more highly expressed in growth-arrested primary cardiomyocytes. Similar to β-galactosidase expression, hTERT had no effect on the increase in p16^{INK4A} that occurred with increasing passage (Fig. 5, lanes 4 and 6).

HFC growth-arrest is independent of telomere shortening

Cellular senescence commonly occurs as a result of telomere shortening during DNA replication. In primary human diploid fibroblasts, the best-studied model of senescence, telomere erosion occurs over more than 40 cell divisions before growth arrest due to senescence (Harley *et al*., 1990). As the cardiomyocyte cultures undergo growth arrest after only 20–22 PDs, we were particularly interested in examining telomere length dynamics in these cells. Samples of genomic DNA from early- (actively dividing) and late- (growth-arrested) passage primary cardiomyocytes and telomerase-infected cardiomyocytes were isolated for telomere length analysis by Southern blot (Fig. 6). The telomere lengths of maximally expanded primary HFCs (Lane 4) were not found to be substantially shorter than those of proliferating early-passage cells (Lane 3). Interestingly, telomeres of hTERT-positive cells (Lanes 5 and 6) were elongated in comparison with cells that had not been infected with virus-expressing telomerase (Lanes 3 and 4), and no difference in average telomere length between proliferating and growth-arrested telomerase-infected samples was observed (Lanes 5 and 6). Thus, increasing the telomere length had no effect on the kinetics of growth of HFCs.

Fig. 5 Western blot showing levels of p16^{INK4A} expression. Lane 1: Saos2 cell line, positive control for p16^{INK4A}; Lane 2: K562 cell line, negative control for p16^{INK4A}; Lane 3: Proliferating HFC cells (4 PD); Lane 4: growth-arrested HFC cells (22 PD); Lane 5: proliferating telomerase-positive HFC cells (4 PD); Lane 6: growth-arrested telomerase-positive HFC cells (22 PD). β-actin was used with each cell type as a loading control.

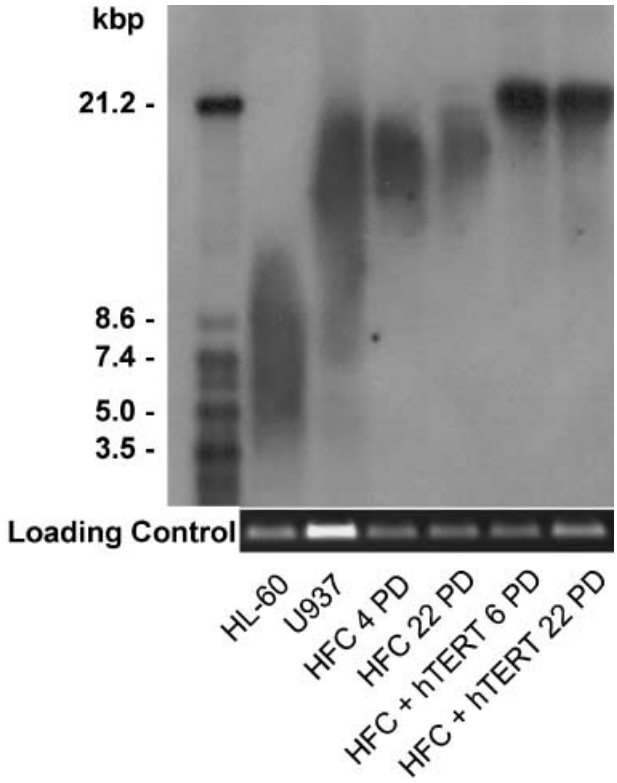

Fig. 6 Southern blot comparing telomere lengths in HFC cells with and without telomerase. Lane 1: HL-60 cell line, control for short telomeres; Lane 2: U937 cell line, control for long telomeres; Lane 3: proliferating HFC cells (4 PD); Lane 4: growth-arrested HFC cells (22 PD); Lane 5: proliferating telomerase-positive HFC cells (6 PD); Lane 6: growth-arrested telomerase-positive HFC cells (22 PD).

HFC growth-arrest is not prevented by culture in low oxygen conditions

Under normal conditions, cell culture is performed at ambient oxygen levels (20%), considerably higher than the levels thought to exist in the human heart (Winegrad *et al*., 1999). Studies have shown that in certain cell types ambient oxygen induces oxidative stress and limits cell growth (Busuttil *et al*., 2003; Parrinello *et al*., 2003; Martin *et al*., 2004). To examine whether oxidative stress was underlying the growth arrest

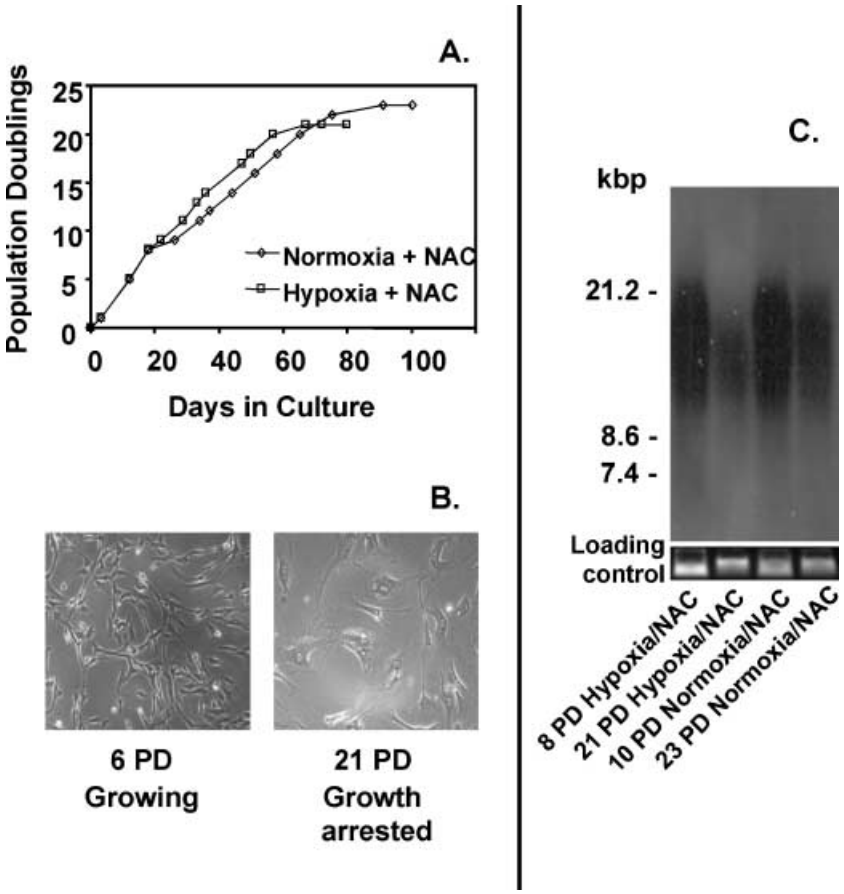

Fig. 7 (A) *In vitro* growth of human fetal cardiomyocyte populations. Number of PDs are plotted as a function of time in culture. Diamonds represent cells cultured in the presence of 20% oxygen plus 1 mM *N*-acetyl cysteine. Squares represent cells cultured in the presence of 1% oxygen plus 1 mM *N*-acetyl cysteine. (B) Phase contrast images of proliferating (6 PD) and growth-arrested (21 PD) cells cultured in the presence of 1% oxygen plus 1 mM *N*-acetyl cysteine. (C) Southern blot comparing telomere lengths in HFC cells cultured in the presence of different oxygen concentrations. Lanes 1 and 2: proliferating (8 PD) and growth-arrested (21 PD) cells cultured in the presence of 1% oxygen plus 1 mM *N*-acetyl cysteine; Lanes 3 and 4: proliferating (10 PD) and growth-arrested cells (23 PD) cultured in the presence of 20% oxygen plus 1 mM *N*-acetyl cysteine.

observed in primary HFC, we cultured these cells in the continuous presence of an oxygen concentration of 1% – a level that is within the physiologic range for cardiomyocytes (Roy *et al*., 2003). To reduce the level of oxidative stress further, tissue culture medium was supplemented with 1 mM *N*-acetyl cysteine, a free radical scavenger that extends lifespan in some cells (Haendeler *et al*., 2004). *N*-acetyl cysteine and 1% oxygen were unable to prolong the lifespan of primary HFC. As with cells cultured in the presence of ambient oxygen (normoxia), cells cultured in the presence of 1% oxygen (hypoxia) growth-arrested after 20–24 PDs (Fig. 7A). Growth-arrested cells showed the enlarged, flattened morphology characteristic of senescent cells (Fig. 7B), and Southern blot analysis revealed that culture of cells in the presence of hypoxic conditions and a free radical scavenger had no beneficial protective effect on telomere length in these cells (Fig. 7C).

Transcript levels of Bmi-1 decrease in senescent cardiomyocytes

The Polycomb gene Bmi-1 is a transcriptional repressor of the INK4A locus (Jacobs *et al*., 1999), which in humans encodes the tumor suppressor proteins p16 and $p14^{ARF}$. In human lung (WI-38) fibroblasts, overexpression of Bmi-1 increases replicative lifespan by suppressing the $p16^{INK4A}$ senescence pathway (Itahana *et al*., 2003). Conversely, senescence in human WI-38 fibroblasts is accompanied by a decline in endogenous Bmi-1 levels, suggesting that Bmi-1 contributes to the senescence program in these cells (Itahana *et al*., 2003).

Using a quantitative real-time RT-PCR approach, we measured the expression of Bmi-1 transcripts in proliferating and senescent HFCs in the absence or presence of exogenous telomerase (Fig. 8A). In senescent primary HFCs, Bmi-1 levels were significantly decreased ($P < 0.001$) compared with presenescent cells. Similarly, in HFCs transduced with hTERT, senescent cells showed a significant ($P < 0.001$) decline in Bmi-1 levels when compared with earlier passage presenescent cells (Fig. 8A).

Reduced transcription of Ets and Id in growth-arrested cardiomyocytes

In human diploid fibroblasts, Ets transcription factors have the ability to activate the $p16^{INK4A}$ promoter via an Ets-binding site, and induction of $p16^{INK4A}$ by Ets can be inhibited by direct interaction with an Id helix–loop–helix protein (Ohtani *et al*., 2001; Zebedee & Hara, 2001). Mouse embryo fibroblasts from Id1-deficient mice express high levels of $p16^{INK4A}$ (Lyden *et al*., 1999), and loss of Id function has been linked to replicative senescence in human fibroblasts (Hara *et al*., 1994). To determine whether changes in Ets and/or Ids expression might

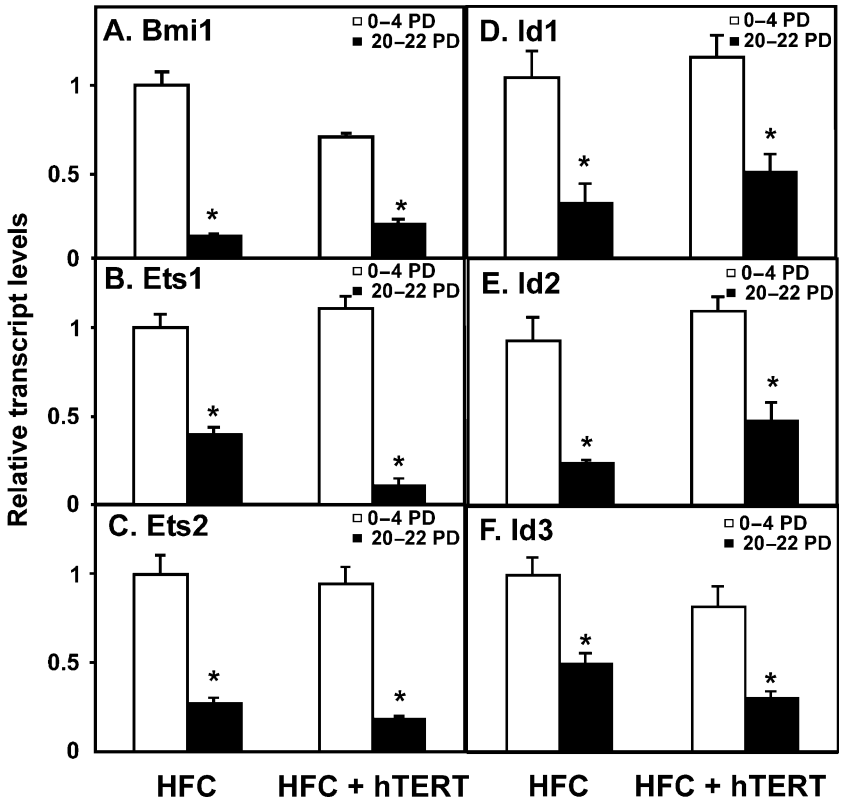

Fig. 8 Quantitative RT-PCR analysis of Bmi-1, Ets and Id transcript levels in proliferating (0–4 PD) and growth-arrested (20–22 PD) HFC cells with and without telomerase. Panel A: Bmi-1; Panel B: Ets1; Panel C: Ets2; Panel D: Id1; Panel E: Id2; Panel F: Id3. Solid bars represent growth-arrested cells. Values shown represent means of six independent samples ± SEM. $*P < 0.001$ when compared with proliferating cells.

accompany $p16^{INK4A}$ expression in senescent HFCs, we utilized quantitative real-time RT-PCR to examine relative expression of Ets1, Ets2, Id1, Id2 and Id3 mRNA transcript levels in proliferating and growth-arrested HFCs in the absence or presence of the hTERT gene (Fig. 8B–F). Each of these genes showed a significant reduction ($P < 0.001$) in transcript expression in growth-arrested cells (20–22 PDs). With each gene studied, this reduction was observed in both primary HFC cells and HFC cells that had been infected with telomerase.

Discussion

Our results indicate that HFCs have a limited proliferative capacity and that cellular senescence is responsible for the proliferative failure that occurs after 20–25 PDs. We have demonstrated that the cellular senescence program underlying growth arrest in cultured human fetal cardiomyocytes is independent of telomerase activity and telomere length, but is accompanied by increased $p16^{INK4A}$ and β-galactosidase expression along with a reduction in the expression of Bmi-1, Ets and Id genes.

Cellular senescence occurs in human somatic cells grown in culture following a limited number of PDs, and is accompanied by growth arrest, morphological changes and increased expression of the $p16^{INK4A}$ tumor suppressor protein (Duncan *et al*., 2000) and senescence-associated markers such as SA β-galactosidase. The most commonly described model of senescence proposes that the process is due to telomere shortening. Expression of exogenous TERT has been shown to restore telomere lengths and extend cellular lifespan (Bodnar *et al*., 1998; Halvorsen *et al*., 1999; Duncan *et al*., 2000). In some cases, such as primary human fibroblasts, TERT is sufficient to immortalize cells without causing transformation or compromising genomic stability (Jiang *et al*., 1999; Vaziri *et al*., 1999). Although telomere length plays a major role in senescence in many cell types, additional factors are clearly involved. For instance, senescence in human keratinocytes is associated with loss of telomerase activity without shortening of telomeres (Kang *et al*., 1998). In astrocytes, a proliferative lifespan barrier exists that is independent of telomere length but is associated with increased SA β-galactosidase and $p16^{INK4A}$ (Evans *et al*., 2003). In human pancreatic beta-cells, growth arrest is accompanied by extremely rapid shortening of telomeres and increased expression of SA β-galactosidase and $p16^{INK4A}$. However, the senescent phenotype was not prevented by retroviral induction of the hTERT gene, despite telomerase activity being induced (Halvorsen *et al*., 2000). Taken together, these data indicate that telomere-length-independent mechanisms can induce senescence in human somatic cells, and that different cell types may utilize different senescence pathways.

To determine whether the limited proliferative ability of primary fetal cardiomyocytes was caused by a lack of telomerase activity, we examined the effects of exogenous telomerase upon lifespan of these cells using a retroviral hTERT vector which has been previously demonstrated to extend the proliferative lifespan of primary human fibroblasts and immortalize human pancreatic cell lines (Halvorsen *et al*., 1999). We found that

transduction of the hTERT gene and induction of telomerase activity had no observable effect on the proliferative lifespan of human fetal cardiomyocytes, despite a clear telomeric elongation mediated by hTERT. This suggests that additional signals other than telomere shortening may activate or enforce the HFC senescence program and that the replicative fate of cultured HFCs is not determined simply by a mitotic clock of telomere length.

In common with many telomere-dependent and -independent models of senescence, we observed a marked up-regulation of $p16^{INK4A}$ expression in growth-arrested fetal cardiomyocytes. This up-regulation was observed in all senescent cultures studied, whether with or without induced telomerase activity. These findings are in keeping with recent reports showing increased levels of $p16^{INK4A}$ in cardiomyocytes from aging mice (Torella *et al*., 2004) and in aging diseased human hearts (Chimenti *et al*., 2003). However, the *in vivo* studies found that cardiomyocyte senescence is accompanied by telomere shortening, in contrast to our *in vitro* data.

Of all the characteristics of senescent cells, up-regulation of $p16^{INK4A}$ seems to be the most universal. We have shown that HFCs can have their lifespan significantly extended by forced expression of a mutant CDK4 that inhibits the binding of $p16^{INK4A}$ (Ball *et al*., 2004). These cells eventually senesce accompanied by telomere shortening, suggesting that the senescence program reported here in primary HFC cells is $p16^{INK4A}$-mediated. Thus, understanding the inputs into $p16^{INK4A}$ gene expression may provide important insights into the regulation of HFC senescence. The INK4A locus is known to be repressed by the Polycomb protein Bmi-1 (Jacobs *et al*., 1999). A recent study in human WI-38 fibroblasts reported that overexpression of Bmi-1 rescues these cells from extrinsic (telomere-independent) senescence and extends their replicative lifespan by repressing p16 (Itahana *et al*., 2003). We used quantitative RT-PCR to examine Bmi-1 transcripts in proliferating and senescent HFCs with or without telomerase and found that senescence was accompanied by a marked decline in Bmi-1 levels, concomitant with the increased expression of $p16^{INK4A}$. Thus it appears that the telomere-independent senescence observed in cultured HFCs may in part be mediated by a decline in Bmi-1 levels and that the consequent decline in repression of the INK4A locus leads to the increased expression of p16 observed in senescent HFCs.

In addition to Bmi-1, Ets-domain and Id helix–loop–helix transcription factor proteins (Benezra *et al*., 1990; Sharrocks, 2001) are thought to play a role in regulating $p16^{INK4A}$ expression, and ectopic expression of Id1, 2 and 3 has been shown to increase the lifespan of human keratinocytes (Alani *et al*., 1999; Nickoloff *et al*., 2000). We examined Ets1, Ets2, Id1, Id2 and Id3 expression in senescent cardiomyocytes. Levels of each were found to be significantly reduced in senescent cells, and expression of exogenous telomerase had no effect on Id or Ets expression. These data suggest a possible role for Ets and Ids in mediating $p16^{INK4A}$ expression in aging cardiomyocytes. The reduction in Id expression is consistent with the roles defined for these in other cell types. However, the reduction in both Ets1 and Ets2 differs from observations in senescent human fibroblasts, where Ets factors increase in senescent cells (Ohtani *et al*., 2001). It is important to note that the Ets family of transcription factors comprises many members (Sharrocks, 2001), and it is conceivable that different members may increase in expression to regulate $p16^{INK4A}$ in different cell types. Our findings provide the first evidence of a correlation between Bmi-1, Ets, Id and increased $p16^{INK4A}$ expression in cardiomyocytes, and may provide a basis for studies examining the nature of the increased $p16^{INK4A}$ expression observed in aging and diseased human heart.

In addition to shedding new light on the behavior of fetal cardiomyocytes in culture, this study has implications for the development of a cell-based therapy for cardiac injury using primary fetal heart tissue. If transplantation of fetal cardiomyocytes is to become clinically effective in the treatment of heart failure, an approach to bypassing cellular senescence must be found.

Experimental procedures

Isolation and culture of human fetal heart cells

Ventricular cardiomyocytes from human fetal hearts of 22–24 weeks gestation (Advanced Bioscience Resources) were isolated by collagenase digestion of ventricular tissue followed by Percoll gradient centrifugation (Goldman & Wurzel, 1992). Cell cultures were maintained in a humidified environment in the presence of 10% CO_2 and atmospheric (20%) O_2. Cultures were fed with Dulbecco's modified Eagle medium with high glucose and 10% fetal bovine serum every 3 days, and subcultured by trypsinization when cells neared confluence. At each passage, cell number was determined and PD values calculated. In studies examining the role of reduced O_2 levels upon proliferative lifespan, cells were cultured continuously in a Ruskinn INVIVO$_2$ 400 anaerobic workstation maintaining 1% O_2.

Infection with hTERT retroviral vector

Isolated ventricular cardiomyocytes were infected after 1 PD with an hTERT-expressing retroviral vector (Halvorsen *et al*., 1999) and selected in 100 μg mL^{-1} hygromycin. A resistant hTERT-positive population was obtained and expanded in the same manner as primary ventricular cardiomyocytes.

Immunocytochemistry

Immunostaining was performed on cell monolayers fixed in 4% paraformaldehyde. Primary antibodies were monoclonal anti-α-actinin (sarcomeric) clone EA-53 (Sigma) diluted 1 : 200 and monoclonal MF20, which recognizes sarcomeric myosins, diluted 1 : 100. MF20 was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, USA. Secondary antibody was fluorophore-labeled donkey anti-mouse (Alexa Fluor 488, Molecular Probes, Inc.), used at a 1 : 250 dilution. Nuclear

counterstaining was performed with 300 nmol L^{-1} 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Inc.) in PBS.

β-Galactosidase staining

Monolayer cell cultures were washed with PBS, then fixed for 15 min at room temperature with 1.25% glutaraldehyde in PBS. Senescence-associated galactosidase activity was localized as described previously (Halvorsen *et al*., 2000).

RT-PCR for cardiomyocyte markers

RT-PCR was performed as described previously (Dufayet de la Tour *et al*., 2001). With each primer set, PCR amplification conditions were 5 min at 94 °C, followed by 40 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s. Primer sequences are shown in Table 1. All primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/primer3 °code.html), and purchased from Proligo (La Jolla, CA, USA). Quantitative RT-PCR reactions (for Bmi-1, Ets1, Ets2, Id1, Id2 and Id3) were performed using the Opticon Real-Time System (MJ Research, San Francisco, CA, USA), using SYBR Green I fluorescent dye for detection. GAPDH was used as a reporter gene for data normalization. Data were analysed using Opticon Monitor software.

Table 1 PCR primers used in this study

Gene product	Primers	Product size (bp)
Gata4	GACGGGTCACTATCTGTGCAAC	475
	AGACATCGCACTGACTGAGAAC	
Nkx2.5	GGTGGAGCTGGAGAAGACAG	197
	AGATCTTGACCTGCGTGGAC	
Tbx5	ACCACAAACCAACCTTGAGC	190
	GCAAGGTTCTGCTCTCCAAC	
MLC-2v	ACAGGGATGGCTTCATTGAC	192
	ATGCGTTGAGAATGGTTTCC	
Titin	CAAAGCGTTGTGGTACTGGA	418
	GCTCTTCCAACGCTATTGGT	
cTNT	AGTGGGAAGAGGCAGACTGA	154
	CGAACTTCTCTGCCTCCAAG	
Cd31	TCCCACGCCAAAATGTTAAGTGAG	405
	GATCAAGAGAGCAATGATCACTCC	
vWF	AGTTCATGGAGGAGGTGATTCAGC	540
	AGCCATCCAGGAGAAGGATCACG	
Bmi-1	TCATCCTTCTGCTGATGCTG	221
	GCATCACAGTCATTGCTGCT	
Ets1	TGGAGTCAACCCAGCCTATC	233
	CGTACGGGATGGTAGCAAGT	
Ets2	GCAAGGCTGTGATGAGTCAA	170
	CCTCTGCAGATTCACGTTCA	
Id1	AAACGTGCTGCTCTACGACA	153
	GATTCCGAGTTCAGCTCCAA	
Id2	CGTGAGGTCCGTTAGGAAAA	246
	AGGCTGACAATAGTGGGATG	
Id3	ACTCAGCTTAGCCAGGTGGA	167
	AAGCTCCTTTTGTCGTTGGA	
Gapdh	CTCTGCTCCTCCTGTTCGAC	187
	AATGAAGGGGTCATTGATGG	

TRAP assay

Quantitative determination of telomerase activity was performed using the TRAP procedure with the TeloTAGGG telomerase PCR ELISA kit (Roche) according to the manufacturer's instructions. Integrity of assay was determined by using heat-inactivated samples as negative controls and telomerase-expressing 293 cell samples as positive controls.

Telomere length assay

Telomere length was determined by Southern blot analysis of terminal restriction fragments obtained by the digestion of genomic DNA, using a TeloTAGGG Telomere Length Assay kit (Roche). Briefly, genomic DNA was isolated then digested with *Rsa*I/*Hin*fI, separated on a 0.8% agarose gel, transferred to a positively charged nylon membrane (Roche) and hybridized with a telomere-specific, DIG-labeled probe, which was incubated with anti-DIG-alkaline phosphatase and detected by chemiluminescence.

Western blot for $p16^{INK4A}$

Cells from monolayer cultures were lysed using RIPA buffer (Boston Bioproducts) containing a protease inhibitor cocktail (Calbiochem). Expression of $p16^{INK4A}$ was determined by Western blot analysis of 10 μg protein with an anti-human $p16^{INK4A}$ monoclonal antibody (Pharmingen, San Diego, CA, USA). The same membrane was reprobed with anti-actin rabbit antibody A2066 (Sigma) to verify that equivalent amounts of protein were loaded onto each lane. Bound antibody was detected with horseradish peroxidase-linked anti-mouse or anti-rabbit Ig (Amersham Pharmacia Biotech, Little Chalfont, UK) and enhanced chemiluminescence (Amersham Pharmacia Biotech).

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM) for a given number of observations (*n*). Groups of data were compared using unpaired Student's *t*-test.

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