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Gene expression and regulation in H_2O_2-induced premature senescence of human foreskin fibroblasts expressing or not telomerase

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Abstract

We compared the DNA-binding activity of transcription factors and gene expression patterns in BJ human diploid fibroblasts (HDFs) expressing or not telomerase (hTERT) in stress-induced premature senescence (SIPS). Senescent BJ cells were also studied. Hydrogen peroxide (H_2O_2)-induced SIPS modulated gene expression in both BJ and hTERT-BJ1 cells. Increased $p21^{WAF-1}$ mRNA level was amongst the common gene expression changes in BJ and hTERT-BJ1 cells induced by SIPS. Telomerase expression markedly changed gene expression in non-stressful conditions. Expression patterns of senescent BJ cells partially overlapped those of BJ and hTERT-BJ1 cells in SIPS. The basal levels of DNA-binding activity of NF-κB and phosphorylated ATF-2 were different in BJ and hTERT-BJ1 cells. Both cell lines displayed a higher DNA-binding activity of p53 and HIF-1 72 h after H_2O_2 exposure. Our results indicate that similar mechanisms involving $p21^{WAF-1}$ and probably p53 are at work in BJ and hTERT-BJ1 HDFs under H_2O_2-induced SIPS, suggesting that generalized DNA damage rather than telomere length/telomerase plays a crucial role in H_2O_2-induced SIPS. We propose that H_2O_2-induced SIPS involves a rearrangement of proliferative and apoptotic pathways. The marked changes in gene expression induced by telomerase suggest that apart from immortalization of HDFs, telomerase also alters the normal cellular functions but does not protect against SIPS.

Keywords: Cellular senescence; Fibroblasts; Gene expression; H_2O_2; Telomerase; Telomeres

1. Introduction

Normal human diploid fibroblasts (HDFs) stop dividing after a certain number of population doublings (PDs) in vitro (Hayflick and Moorhead, 1961). This phenomenon is termed replicative senescence (RS). Telomere shortening observed at each cell division eventually leads telomeres to critical lengths. Critically short telomeres are probably recognized as DNA damage and activate p53, which triggers growth arrest through the overexpression of cyclin-dependent kinase inhibitors such as $p21^{WAF-1}$. $p21^{WAF-1}$ in turn inhibits the phosphorylation of the retinoblastoma protein (Vaziri and Benchimol, 1996). Telomerase is a reverse-transcriptase enzyme that elongates the telomeres. HDFs transfected with the catalytic subunit of human telomerase (hTERT), although they are not transformed, do not display RS when considered as cell populations (Bodnar et al., 1998). Recent data, however, indicates that high levels of telomerase expression could favor RS of a small fraction of the cell population of HDFs (Gorbunova et al., 2003).

Stress-induced premature senescence (SIPS) establishes several days after exposure of HDFs to subcytotoxic concentrations of numerous types of oxidants and DNA-damaging agents such as hydrogen peroxide (H_2O_2) or UV radiation. HDFs in SIPS display features common with RS: a senescent morphology, senescence-associated β-galatosidase (SA β-gal) activity, growth arrest in the G1 phase of the cell cycle, etc. (Chen et al., 1998, 2000). Oxidative stress can increase the shortening of telomeres in HDFs. WI-38 HDFs exposed to 40% hyperoxia undergo a mean telomere shortening of 500 bp/PD, compared to the 90 bp/PD normal shortening observed under 20% atmospheric O_2.

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Such accelerated shortening rapidly leads the telomeres to their critical length, triggering irreversible growth arrest of the HDFs (von Zglinicki et al., 1995). Yet, H_2O_2 can induce SIPS without critical shortening of the telomeres. After exposure to subcytotoxic H_2O_2 stress, BJ foreskin HDFs expressing hTERT display features of senescent cells at levels comparable to the wild type BJ HDFs exposed to the same concentration of H_2O_2. Only a slight telomere shortening is observed in both BJ and hTERT-BJ1 HDFs (de Magalhaes et al., 2002; Gorbunova et al., 2002; Matuoka and Chen, 2002) and so the role of the telomeres in SIPS remains unclear.

We used a low-density DNA array representing genes of general interest in cell biology to characterize gene expression of BJ and hTERT-BJ1 HDFs in H_2O_2-induced SIPS. Our interest was to identify changes in gene expression in BJ cells in SIPS and to know whether similar changes exist in BJ cells expressing hTERT. Furthermore, we studied gene expression in senescent BJ HDFs. The excellent reproducibility and validity of the data obtained with low-density DNA arrays technically identical to those employed in the present report was previously demonstrated in terms of chemistry of covalent binding of DNA on activated glass support, optimized length and sequence, hybridization, and statistical analysis (de Longueville et al., 2002). Lastly, and in order to help understand the changes in gene expression, we studied the DNA-binding activity of several transcription factors.

2. Materials and methods

2.1. Cell culture, induction of SIPS by H_2O_2, and isolation of mRNA

hTERT-BJ1 HDFs obtained from Clontech (#C4000-1, Palo Alto, CA, USA) at 111 PD were exposed to H_2O_2 between PD 130 and 150. BJ HDFs were a kind gift from Dr E.E. Medrano, Baylor College, Houston, TX, USA and were stressed between PD 20 and 35. Both these cell lines have been cultured in our laboratory for 3 years and previous reports indicated that the only consequence of hTERT transfection was immortalization (Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999). Both BJ and hTERT-BJ1 cells were cultivated in DMEM medium + 10% fetal calf serum under the classical conditions previously described (Hayflick and Moorhead, 1961). H_2O_2 at 1200 μM diluted in medium with 10% serum for 2 h was previously shown to be subcytotoxic and trigger SIPS in both BJ cell lines (de Magalhaes et al., 2002). Control cultures at the same PD followed the same schedule of medium changes without exposure to exogenous H_2O_2. At 72 h after stress, mRNA was isolated (FastTrack 2.0 mRNA isolation kit, Invitrogen, Carlsbad, CA, USA). Seventy-two hours after subcytotoxic H_2O_2 stress is an optimal time to study H_2O_2-induced SIPS at least in WI-38, IMR-90, BJ, and hTERT-BJ1 HDFs (Frippiat et al., 2001; de Magalhaes et al., 2002; Gorbunova et al., 2002).

2.2. Low-density DNA array design, synthesis of labeled DNA, and hybridization conditions

The DualChip Human General design (Eppendorf, Hamburg, Germany) is based on a system with two arrays per glass slide and the same three sub-arrays per array (triplicate spots per array). The array represents a range of 202 genes involved in basic cellular processes such as metabolism, apoptosis, cell cycle, stress response, pro-inflammatory state and transcription (Supplementary Material). The sequences of the DNA covalently linked to the glass slide were carefully chosen by sequence comparison and it was checked experimentally that no cross-hybridization takes place. Several positive and negative hybridization controls plus detection controls were spotted on the array in order to control the reliability of the experimental data. 0.5 μg of each sample of mRNA was retrotranscribed into DNA according to the manufacturer's instructions. Three synthetic poly(A) + tailed RNA standards were spiked at three different amounts (10, 1 and 0.1 ng/reaction) into the purified mRNA as required by the array kit (Eppendorf, Hamburg, Germany). Normalization of data was possible thanks to three internal standard controls described above and eight housekeeping genes. Triplicates from three independent experiments were performed, meaning hybridizations on nine sub-arrays. The DualChip Human General hybridization was carried out according to the manufacturer's instructions as reported (de Longueville et al., 2002). Detection was performed using a Cy3-conjugated IgG anti-biotin (Jackson Immuno Research Laboratories, West Grove, PA, USA).

2.3. Imaging, statistical analysis, and clustering

Fluorescence of the hybridized arrays was scanned using the Packard ScanArray (Perkin–Elmer, Boston, MA, USA) at a resolution of 10 μm. To maximize the dynamic range of detection, the same arrays were scanned at different photomultiplier gains for quantifying both the high- and low-copy expressed genes. The scanned 16-bit images were imported into the ImaGene 4.1 software (BioDiscovery, Los Angeles, CA, USA) to quantify the signal intensities. The fluorescence intensity of each DNA spot (average of intensity of each pixel present within the spot) was calculated using local mean background subtraction. A signal was accepted if the average intensity after background subtraction was at least 2.5-fold higher than their local background. The three intensity values of the triplicate DNA spots were averaged and used to calculate the intensity ratio between the reference and the test samples.

The data were normalized in two steps. First, the values were corrected using a factor calculated from the intensity ratios of the internal standards in the references and test

samples. The presence of the three internal standards probes at two different locations of the array allowed a measurement of local background and evaluation of the array homogeneity, which is considered in the normalization. However, since the internal standard control does not take into account the purity and quality of the mRNA, a second step of normalization was performed based on expression levels of the housekeeping genes. This process involves calculating the average intensity for a set of housekeeping genes. The variance of the normalized set of housekeeping genes is used to generate an estimate of expected variance, leading to a predicted confidence interval for testing the significance of the ratios obtained. Ratios outside the 95% confidence interval were determined to be significantly different, according to the Chen–Dougherty–Bittner procedure (Chen et al., 1997; reviewed by de Longueville et al., 2002).

2.4. Real time RT-PCR

At 72 h after the last stress, total RNA was extracted from three independent cultures using the Total RNAgent extraction kit (Promega, USA). Total RNA (2 μg) was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, UK). Amplification reaction assays contained 1x SYBR Green PCR Mastermix and primers (Applied Biosystems, The Netherlands) at optimal concentrations. A start at 95 °C for 5 min was followed by 40 cycles at 95 °C for 15 s and 65 °C for 1 min using the 7000 SDS thermal cycler (Applied Biosystems, The Netherlands).

2.5. ELISA kits for detection of transcription factor DNA-binding activity

The ELISA kits DNA-binding assays are based on the use of multi-well plates coated with a cold oligonucleotide containing the consensus binding site for the transcription factor under study. The presence of the DNA-bound transcription factor is then detected by specific antibodies and revealed by colorimetry. The specificity and high reproducibility of this type of assay has been previously demonstrated. In fact, when compared with electrophoretic mobility shift assay, this ELISA assay has been shown to be more sensitive (Renard et al., 2001). Other laboratories also assessed the reproducibility of these ELISA kits in measuring the DNA-binding activity of HIF-1 (Leonard et al., 2003).

Nuclear extracts were obtained from BJ and hTERT-BJ1 cells exposed or not to a single H_2O_2 stress of 1200 μM at 2, 6, 24, 48, and 72 h after the stress. We then determined the DNA-binding activity of p53, HIF-1, CREB, AP-1, NF-κB, ATF-2, PPARg, and NFAT using the TransAM kits (ActiveMotif, San Diego, CA, USA), according to manufacturer's instructions. Triplicates were always performed from three independent experiments.

3. Results

3.1. Gene expression varies in H_2O_2-induced SIPS in BJ ± ectopic hTERT HDFs

BJ and hTERT-BJ1 human diploid skin fibroblasts were exposed to a 2 h-stress with H_2O_2 at 1200 μM, which represented subcytotoxic conditions. At 72 h after stress, both cell lines displayed a large increase in the proportion of the SA β-gal positive cells, a sustained decrease in incorporation of [^{3}H]-thymidine into their DNA, and increased protein levels of $p21^{WAF-1}$ (de Magalhaes et al., 2002). The low-density DNA array DualChip Human General was used to study the mRNA levels of 202 preselected genes (Supplementary Material, Fig. 1) in BJ and hTERT-BJ1 HDFs in H_2O_2-induced SIPS.

At 72 h after H_2O_2 stress, 21 genes were found to be differentially expressed in BJ cells, with 14 genes displaying an increased and seven genes a decreased mRNA steady-state level. In hTERT-BJ1 cells in H_2O_2-induced SIPS, nine genes displayed an increased while seven genes displayed a decreased mRNA steady-state level. Thirty-four genes were differentially expressed in BJ cells at late PDs when

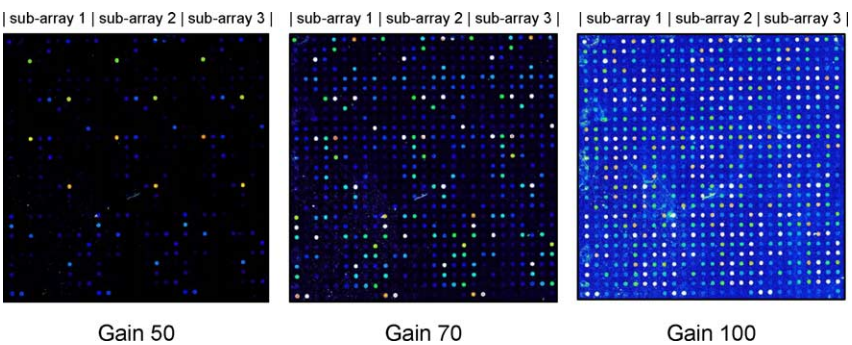

Fig. 1. Representative example of images obtained with DualChip scanned at different photomultiplier gains. Each DualChip features triplicate spots (sub-arrays) on each array. Three DualChip were used in each experiment.

Table 1
Correspondence between real-time RT-PCR and the DualChip Human General

Gene	Real-time RT-PCR (%)	Value obtained on DNA array (% of real-time RT-PCR)	Percentage of difference
ApoJ	100	90.4 ± 19.0	−9.6 ± 19
Fibronectin	100	109.8 ± 10.0	+9.8 ± 10
Osteonectin	100	80.5 ± 23.2	−19.1 ± 23.2
SM22	100	91.7 ± 0	−8.3 ± 0
p21	100	85.7 ± 20.0	−14.3 ± 20.0
p53	100	100.0 ± 0	0

Values obtained with real-time RT-PCR were considered as reference (100%) and compared to the values obtained using the DualChip Human General. Results are expressed in percentage. ± represents the variation of the results obtained with independent cell cultures which considers both technical and experimental variability. Since this compared variability is sufficient for a statistical analysis we did not measure the technical variability alone, which was anyway already deeply analyzed by the Eppendorf software (de Longueville et al., 2002).

compared to young BJ cells. Lastly, 54 genes were also differentially expressed in hTERT-BJ1 compared to BJ cells.

The correspondence between real-time RT-PCR and this array's data has been checked for several differentially expressed genes including: APOJ, FN1, MMP-1, MMP-2, p21, and SM22 (Table 1). Other genes such as FOS, ON, and p53 were also verified though these were not differentially expressed in our experimental conditions. The variation of the results obtained with independent cell cultures was also very acceptable (Table 1). Although mRNA and protein levels may not always correlate (Gygi et al., 1999), changes in gene expression represent a perturbation of the system under study. Our gene expression results yield an insight at the regulatory level but further analysis of protein or enzymatic activities is necessary to understand functional changes in these pathways.

3.2. Changes in gene expression triggered by hTERT

Nineteen genes displayed an increased- and 35 genes displayed a decreased-mRNA steady-state level in hTERT-BJ1 when compared to BJ cells (Table 2). The over-expression of CTGF, VEGF, and FGF2 (growth factors) suggests that telomerase could favor cellular proliferation, reinforcing a recent study on human mammary epithelial cells (Smith et al., 2003). Also in accordance with the results from mammary epithelial cells that suggest telomerase causes resistance to the anti-proliferative effect of transforming growth factor beta, TGFBR2 was underexpressed in hTERT-BJ1 cells.

Yet many other genes are differentially expressed. For example, the anti-oxidant SOD2 is downregulated by telomerase. Downregulation of SOD2, as well as downregulation of BAD, could be related to the increased resistance to apoptosis conferred by hTERT (Gorbunova et al., 2002), since SOD2 may be involved in p53-mediated apoptosis (Drane et al., 2001). IL6, an important gene in differentiation and inflammatory response is also down-regulated. Lastly, IGFBP3 is overexpressed in hTERT-BJ1 HDFs, which is intriguing since IGFBP3 is overexpressed in senescent HDFs (Moerman et al., 1993).

Taken as a whole, these results suggest that telomerase prevents RS and promotes proliferation but disrupts many normal cellular functions.

3.3. Characterization of gene expression in H_2O_2-induced SIPS in BJ and hTERT-BJ1 cells

In BJ cells in H_2O_2-induced SIPS, 14 genes displayed an increased- and seven genes displayed a decreased-mRNA steady-state level (Table 3, Fig. 2A). Several are involved in cellular proliferation, e.g. increases in CTGF, EGR1, FGF2, ODC mRNA levels. The increase in EGR1 level is puzzling since EGR1 has been recently suggested, in mice, to control p53 (Krones-Herzig et al., 2003). Interleukin-1 beta (IL1B), which was previously shown to be overexpressed in senescent BJ HDFs (Shelton et al., 1999), becomes expressed in BJ cells in H_2O_2-induced SIPS.

In hTERT-BJ1 cells, 16 genes were differentially expressed in H_2O_2-induced SIPS (Table 4, Fig. 2B). Noteworthy is the increased mRNA level of $p21^{WAF-1}$ in SIPS of BJ and hTERT-BJ1 cells, which correlates with the results obtained previously at the protein level (de Magalhaes et al., 2002). Also in both cell lines, MMP3 is upregulated as a result of stress. The levels of MMP increase in aged skin, a process exacerbated by sun-exposure (Chung et al., 2001). IGFBP5 is also overexpressed in both cell lines. Since IGFBP5 disturbs the binding of IGF-1, evidence suggests that it favors growth arrest and/or apoptotic pathways (Schneider et al., 2002). One previous study in BJ HDFs showed that senescent cells overexpress IGFGP5 (Shelton et al., 1999). The BCL2-associated X protein gene (BAX) is also upregulated by oxidative stress. BAX forms a heterodimer with BCL2 and functions as an apoptotic activator, mediated by p53. Its increased mRNA level suggests that some apoptotic pathways could be active as a result of a single H_2O_2 stress. MDM2 becomes expressed in BJ cells after stress, which is in contrast with BAX since MDM2 inhibits p53-mediated apoptosis. Also interesting is the downregulation of GADD153 in both cell lines since GADD153 has been previously shown to be involved in stress response (Guyton et al., 1996).

There are many genes differentially expressed between the non-stressed BJ and hTERT-BJ1 cells (Table 2). Therefore, comparisons between both cell lines must be taken with care. Several genes differentially expressed in SIPS of BJ cells but not in hTERT-BJ1 cells were also differentially expressed in non-stressed control hTERT-BJ1 cells when compared to non-stressed BJ cells and inversely

Table 2
Genes differentially expressed in hTERT-BJ1 HDFs when compared to normal BJ HDFs

Symbol	Gene name	T/C	Function	GenBank
Genes overexpressed as a result of the presence of telomerase				
CTGF	Connective tissue growth factor	↑ 10.7	Cell proliferation	U14750
CAV1	Caveolin-1	↑ 4.0	Signal transduction; endocytosis; potocytosis	NM_001753
IGFBP3	Insulin growth factor binding protein3	↑ 3.6	Signal transduction; disrupts binding of IGF-1	X64875
GADD153	DNA damage inducible transcript3	↑ 3.4	Stress response	S40706
CANX	Calnexin	↑ 3.0	Protein secretion; probably apoptosis	NM_001746
FN1	Fibronectin	↑ 3.0	Cell adhesion/migration	X02761
ODC	Ornithine decarboxylase1	↑ 2.4	Cell cycle and proliferation	NM_002539
PKM2	pyruvate-kinase-muscle 2	↑ 2.4	Energetic metabolism	M26252
SM22	Transgelin	↑ 2.2	Muscle development	M95787
MEK1	Mitogen activated protein kinase kinase1	↑ 2.2	Cell proliferation control	L11284
TFAP2C	Transcription factor AP2-gamma	↑ 2.1	Morphology	NM_003222
TPA	Plasminogen activator tissue	↑ 2.0	Cell migration and tissue remodeling	NM_000930
FGF2	fibroblast growth factor 2	↑ 2.0	Cell proliferation	NM_002006
VEGF	Vascular endothelial growth factor	↑ 1.8	Angiogenesis; cell proliferation	AF022375
AOP2	Anti-oxidant-protein2	↑ 1.8	Defense system	NM_004905
SMAD3	SMAD3	↑ 1.8	Signal transduction	U68019
VEGFB	Vascular endothelial growth factor B	↑ 1.7	Angiogenesis; cell proliferation	U43368
SHC	p66-SHC transforming protein1	↑ 1.6	Cell proliferation; apoptosis	U73377
Genes not found expressed in BJ cells				
CATB	Catenin, beta 1	↑	Cell adhesion; transduction signal	NM_001904
Genes underexpressed as a result of the presence of telomerase				
SOD2	Superoxide dismutase2	↓ 18.0	Stress response	NM_000636
IL6	Interleukin 6	↓ 5.6	Immune response	NM000600
APOJ	ApolipoproteinJ	↓ 4.1	Lipid metabolism	J02908
MMP7	Matrix metalloproteinase 7	↓ 4.1	Degradation of extracellular matrix	NM_002423
MYBL2	b-myb	↓ 4.0	Cell cycle control	X13293
CCNF	CyclinF	↓ 3.7	Cell cycle control	NM_001761
MMP2	Matrix metalloproteinase 2	↓ 3.4	Degradation of extracellular matrix	NM_004530
TIMP1	Tissue inhibitor of metalloproteinase1	↓ 3.2	Inhibitor of degradation of extracellular matrix	NM_003254
FES	Feline sarcoma oncogene	↓ 3.0	Cell proliferation	X52192
CCND3	CyclinD3	↓ 3.0	Cell cycle control	NM_001760
CSF1R	Colony stimulating factor 1 receptor	↓ 2.7	Cell proliferation	NM_005211
TFAP2B	Transcription factor AP2-beta	↓ 2.7	Neurogenesis; morphology	X95694
CDK6	Cyclin dependent kinase 6	↓ 2.7	Cell proliferation	NM001259
FGF8	Fibroblast growth factor 8	↓ 2.3	Cell proliferation	U36223
SPRR1B	Cornifin	↓ 2.3	Cell structure	NM_003125
BAD	BCL2-antagonist of cell death	↓ 2.2	Pro-apoptotic	NM_004322
BIN1	Bridging integrator 1	↓ 2.2	Cell cycle control; apoptosis	NM_004305
VWF	Factor von willebrand	↓ 2.1	Role in blood coagulation	NM_000552
E2F2	E2F transcription factor2	↓ 2.1	Cell cycle control; apoptosis	NM_004091
CDH11	Cadherine11	↓ 1.9	Calcium-dependent glycoprotein; Cell adhesion	NM_001797
CTSD	CathepsinD	↓ 1.9	Intracellular degradation and turnover of proteins	NM_001904
H4FM	Histone4 member M consensus	↓ 1.8	Cell cycle	NM_003495
IGF1R	Insulin like growth factor 1 receptor	↓ 1.8	Cell proliferation; anti-apoptotic	NM_000875
MMP14	Matrix metalloproteinase 14	↓ 1.8	Degradation of extracellular matrix	NM_004995
IL11RA	Interleukin 11-receptor-alpha	↓ 1.8	Signal transduction	U32324
CKB	Creatin-kinase-brain	↓ 1.8	Energy homeostasis	M16364
MMP11	Matrix metalloproteinase 11	↓ 1.8	Degradation of extracellular matrix	NM_005940
TGFBR2	TGF-beta-R2	↓ 1.7	Cell proliferation	D50683
MMP15	Matrix metalloproteinase 15	↓ 1.7	Degradation of extracellular matrix	NM_002428
Ki-67	Ki-67	↓ 1.6	Cell proliferation	NM_002417
JUND	Jun D proto-oncogene	↓ 1.6	Transcription factor	NM_005354
Genes not found expressed in hTERT-BJ1 cells				
MMP3	Matrix metalloproteinase 3	↓	Degradation of extracellular matrix	NM_002422
PAI2	Plasminogen activator inhibitor type2	↓	Fibrinolysis; cell cycle	J02685
ESR2	Estrogen receptor beta	↓	Cell–cell signaling	X99101
BCLX	BCLX	↓	Apoptosis	NM_001191

We calculated ratio of the normalized hybridization intensity of hTERT-BJ1 cells and the BJ controls (T/C). Genes not expressed in one condition but whose value in the other condition is higher than the mean value for all genes in the corresponding array are represented as genes not found expressed).

Table 3
Genes differentially expressed in BJ HDFs in H_2O_2-induced SIPS

Gene	Name	S/C	Function	GenBank	hTERT-BJ1 in SIPS
Genes overexpressed in H_2O_2-induced SIPS					
MMP3	Matrix metalloproteinase 3	↑4.5	Degradation of extracellular matrix	NM_002422	+expression
CTGF	Connective tissue growth factor	↑2.7	Cell proliferation	U14750	=
BAX	BCL2-associated X protein	↑2.4	Apoptosis	NM_004324	↑1.6
EGR1	Early growth response1	↑2.4	Transcriptional regulator	NM_001964	=
FGF2	Fibroblast growth factor 2	↑2.3	Cell proliferation	NM_002006	=
P21	Cyclin dependent kinase inhibitor 1A	↑2.1	Cell cycle control	U03106	↑2.1
ODC	Ornithine decarboxylase1	↑1.6	Cell cycle and proliferation	NM_002539	↓1.8
CANX	Calnexin	↑1.6	Protein secretion; probably apoptosis	NM_001746	=
IGFBP5	Insulin growth factor binding protein5	↑1.6	Signal transduction; disrupts binding of IGF-1	M65062	↑2.1
Genes not found expressed in non-stressed BJ fibroblasts					
CATB	Catenin, beta 1	↑	Cell adhesion; transduction signal	NM_001904	=
CASP9	Caspase9	↑	Apoptosis	NM_001229	=
IL1B	Interleukin1 beta	↑	Inflammatory and immune responses	M15330	=
CASP2	Caspase2	↑	Apoptosis	NM_001224	=
MDM2	MDM2	↑	Cell cycle control	NM_002392	=
Genes underexpressed in H_2O_2-induced SIPS					
AOP2	Anti-oxidant-protein2	↓2.6	Defense system	NM_004905	=
TFAP2A	Transcription factor AP2-alpha	↓1.8	Morphology	M36711	↑1.9
VEGFR2	Vascular endothelial growth factor receptor2	↓1.7	Angiogenesis; cell proliferation	NM_002253	=
VEGFR3	Vascular endothelial growth factor receptor3	↓1.6	Angiogenesis; cell proliferation	NM_002020	=
GADD153	DNA damage inducible transcript3	↓1.6	Stress response	S40706	↓4.7
PAI1	Plasminogen activator inhibitor type1	↓1.6	Fibrinolysis; cell cycle	M14083	=
IGF1R	Insulin like growth factor 1 receptor	↓1.5	Cell proliferation; anti-apoptotic	NM_000875	=

We calculated the ratio of the normalized hybridization intensity of BJ cells in H_2O_2-induced SIPS and the BJ controls (S/C). Genes not expressed in one condition but whose value in the other condition is higher than the mean value for all genes in the corresponding array are represented as genes not found expressed.

(Tables 2–4). For example, FGF2, CTGF, and CANX are overexpressed in BJ cells as a result of a single H_2O_2 stress but not in hTERT-BJ1 cells; yet these genes are already overexpressed in BJ cells compared to hTERT-BJ1 controls. Therefore, in those cases, when looking at the effects of the H_2O_2 stress on the gene profiles of BJ or hTERT-BJ1 cells, we witness a converging shift in gene expression.

3.4. Gene expression in senescent BJ cells versus BJ cells at early PD

We also compared young versus old BJ cells (Table 5). As previously mentioned, gene expression changed more between young and old BJ cells than when we submitted young BJ cells to H_2O_2 stress. Although some key genes were common between SIPS and RS (e.g. $p21^{WAF-1}$), the degree of gene expression shift between SIPS and RS suggests that SIPS and RS are not exactly the same process. It remains to be seen which of the processes, RS or SIPS, is predominant in the in vivo degeneration of tissues, namely stress-prone tissues like the skin. MMP3 and MMP1 are upregulated in old BJ cells, as happens in aged or photoaged skin (Chung et al., 2001), perhaps as a result of $p21^{WAF-1}$ activity (Perlman et al., 2003). IGFBP5, PAI2, IL-6, caveolin-1, pyruvate kinase, etc. are also upregulated by senescence, as reported by others (Shelton et al., 1999; Dierick et al., 2002; Volonte et al., 2002; Zhang et al., 2003). CCNF and MYBL2 are downregulated, as has been previously reported (Shelton et al., 1999; Ly et al., 2000). A decrease of 2.1-fold of IGF1-receptor is also worth to be reported, given its potential role in resistance against apoptosis.

3.5. DNA-binding activity of p53, HIF, CREB, AP-1, NF-κB, PPARg, and NFAT

Although DNA arrays offer a picture of the changes that cells undergo during SIPS, they cannot by themselves offer an explanation as to what mechanisms regulate the process. Therefore, we studied the activity of several transcription factors at different times after a single H_2O_2 stress in an attempt to uncover the underlying mechanisms responsible for SIPS. The colorimetric ELISA method used in this work

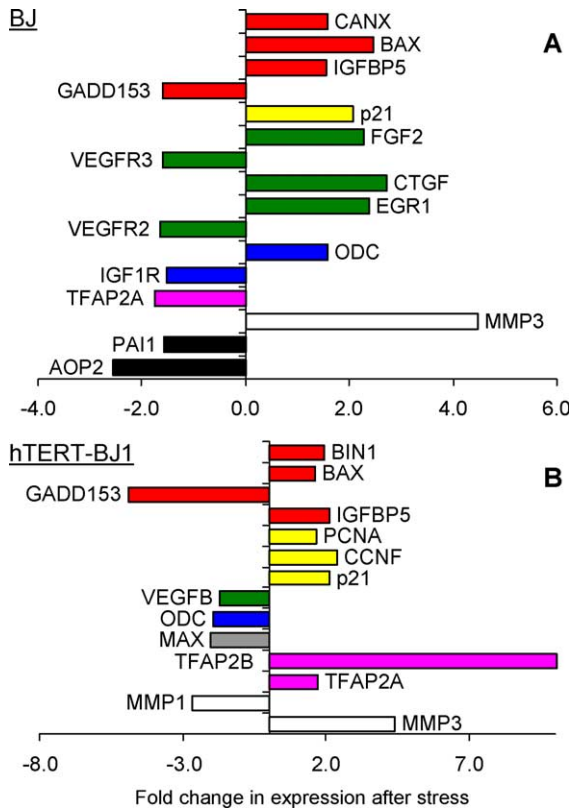

Fig. 2. Genes differentially expressed in BJ (A) and hTERT-BJ1 (B) fibroblasts in H_2O_2-induced SIPS. Values represent the ratio of the normalized hybridization intensity. Each gene has been classified by function according to what best suits its role based on the present literature. Pro-apoptotic = Red; Anti-proliferative = Yellow; Pro-proliferative = Green; Anti-apoptotic = Blue; Morphology = Pink; Pro- or anti-apoptotic depending on context = Gray; Degradation of extracellular matrix = White; Miscellaneous = Black (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

was already compared to mobility shift assays and showed increased sensitivity (Renard et al., 2001).

The DNA-binding activity of p53 increases after a single H_2O_2 stress in both BJ (Fig. 3A) and hTERT-BJ1 cells (Fig. 3B). There appears to be a peak of p53's DNA-binding activity at 2 h after the stress, which stabilizes at 6 h. Interestingly, at 72 h after the stress, p53 activity is still considerably higher (±2-fold) than in control cells. In contrast, p53 protein level upregulation has been previously shown in BJ and hTERT-BJ cells at 24 h after stress (Morales et al., 1999), but not at 72 h (de Magalhaes et al., 2002). In addition, results from IMR-90 fibroblasts showed that p53 upregulation after stress fades in a few days, which led to the conclusion that the role of p53 in SIPS was minor (Chen et al., 1998, 2000). Finally, the basal level of p53 DNA-binding activity is slightly lower in hTERT-BJ1 cells when compared to BJ controls, suggesting that this difference may be responsible for the SOD2 downregulation in hTERT-BJ1 cells (Drane et al., 2001).

It is clear that HIF-1's DNA-binding activity increases as a result of a single H_2O_2 stress, both in BJ (Fig. 3C) and hTERT-BJ1 (Fig. 3D) cells. This data is in agreement with the mitochondrial model of HIF-1 in which oxidative stress activates HIF-1, meaning that H_2O_2 might directly activate HIF-1 (Michiels et al., 2002). The increase in DNA-binding activity of HIF-1 can help explain the overexpression of FGF2 in H_2O_2-induced SIPS since FGF2 levels have been suggested to be mediated by HIF-1 (Li et al., 2002).

Although the DNA-binding activity of NF-κB does not appear to be altered during the 72 h after a single H_2O_2 stress, we noticed that the basal NF-κB DNA-binding activity is at least 2-fold higher in BJ cells compared to hTERT-BJ1 (243 ± 82%). On the contrary, phosphorylated ATF-2's basal DNA-binding activity is much higher in hTERT-BJ1 cells compared to BJ controls (at least 3-fold since the DNA-binding activity increases, 371 ± 60%). Regarding the other studied transcription factors (AP-1, CREB, NFAT, PPARg), we did not find any significant differences in DNA-binding activity either as a result of a single H_2O_2 stress or between BJ and hTERT-BJ1 cells (data not shown).

4. Discussion

It was reported that $p21^{WAF-1}$ is overexpressed at mRNA and protein levels in H_2O_2-induced SIPS in IMR-90 (Chen et al., 1998, 2000) and BJ HDFs (de Magalhaes et al., 2002). $p21^{WAF-1}$ inhibits the phosphorylation of the retinoblastoma protein by cyclin-dependent kinases, explaining the growth arrest observed in SIPS and the appearance of biomarkers of senescence such as SA β-gal, senescent morphology, etc. This overexpression of $p21^{WAF-1}$ can be due to an increased DNA-binding activity of p53, as observed here from 2 to 72 h after stress. Upregulation of p53's protein level had been reported previously at 24 (Morales et al., 1999) but not at 72 h after stress in BJ cells (de Magalhaes et al., 2002). Our DNA array results also did not show any significant change in the steady-state mRNA level of p53 following H_2O_2 stress in either cell line. Yet evidence suggests that post-translational modifications activate p53 in high-passage HDFs (Atadja et al., 1995), explaining why p53's DNA-binding activity, but not its protein or mRNA levels, increases at 72 h after H_2O_2 stress. Although p53-independent induction of $p21^{WAF-1}$ has been reported, $p21^{WAF-1}$ activation due to DNA damage probably involves p53 (Michieli et al., 1994). Nevertheless, a p53-independent induction of $p21^{WAF-1}$ is possible in this type of experimental model since IMR-90 HDFs lacking p53 due to ectopic expression of the viral protein E6 are still able to develop a senescent phenotype after subcytotoxic H_2O_2 stress, while cells lacking E7 cannot, since pRb is necessary for SIPS to occur (Chen et al., 2000). Moreover, the kinase activity of cyclin-dependent kinase 2 has been shown to be

Table 4
Genes differentially expressed in hTERT-BJ1 HDFs in H_2O_2-induced SIPS

Symbol	Gene name	S/C	Function	GenBank	BJ in SIPS
Genes overexpressed in H_2O_2-induced SIPS					
TFAP2B	Transcription factor AP2-beta	↑ 13.6	Neurogenesis; morphology	X95694	=
CCNF	CyclinF	↑ 3.1	Cell cycle control	NM_001761	=
p21	Cyclin dependent kinase inhibitor 1A	↑ 2.1	Cell cycle control	U03106	↑ 2.1
IGFBP5	Insulin growth factor binding protein5	↑ 2.1	Signal transduction; disrupts binding of IGF-1	M65062	↑ 1.6
TFAP2A	Transcription factor AP2-alpha	↑ 1.9	Morphology	M36711	↓ 1.8
BIN1	Bridging integrator 1	↑ 1.8	Cell cycle control; apoptosis	NM_004305	=
PCNA	Proliferating cell nuclear antigen	↑ 1.6	Control of DNA replication	NM002592	=
BAX	BCL2-associated X protein	↑ 1.6	Apoptosis	NM_004324	↑ 2.4
Genes not found expressed in non-stressed hTERT-BJ1 fibroblasts					
MMP3	Matrix metalloproteinase 3	↑	Degradation of extracellular matrix	NM_002422	↑ 4.5
Genes underexpressed in H_2O_2-induced SIPS					
GADD153	DNA damage inducible transcript3	↓ 4.7	Stress response	S40706	↓ 1.6
MMP1	Matrix metalloproteinase 1	↓ 2.8	Degradation of extracellular matrix	NM_002421	=
MAX	MAX protein	↓ 2.2	Cell proliferation control	NM_002382	=
ODC	Ornithine decarboxylase1	↓ 1.8	Cell cycle and proliferation	NM_002539	↑ 1.6
VEGFB	Vascular endothelial growth factor B	↓ 1.6	Angiogenesis; cell proliferation	U43368	=
Genes not found expressed in hTERT-BJ1 cells in H_2O_2-induced SIPS					
HMOX	Heme-oxygenase	↓	Defense system	NM_002133	=
KNSL6	Mitotic-centromere-associated-kinesin	↓	Cell proliferation	NM_006845	=

We calculated the ratio of the normalized hybridization intensity of hTERT-BJ1 cells in H_2O_2-induced SIPS and the respective controls (S/C). Genes not expressed in one condition but whose value in the other condition is higher than the mean value for all genes in the corresponding array are represented as genes not found expressed.

greatly decreased in a $p21^{WAF-1}$-independent manner in IMR-90 cells (Frippiat et al., 2003), suggesting other players may be involved. For instance, the ATM, ATR, and DNA-PK proteins are possible players in initiating SIPS (Yang et al., 2003).

In accordance with previous results (de Magalhaes et al., 2002; Gorbunova et al., 2002; Matuoka and Chen, 2002), the expression of telomerase does not appear to protect or interfere with the appearance of SIPS or its general mechanisms occurring through $p21^{WAF-1}$ overexpression. One plausible explanation is that disruption of the telomeres, rather than telomere shortening, induces SIPS. Indeed, exposure to oligonucleotides homologous to the telomere 3′-overhang sequence induces a senescent phenotype (Li et al., 2003). Yet telomerase appears to stabilize the telomeres (Blackburn, 2000). The inability of telomerase to interfere with SIPS suggests generalized DNA damage generated by H_2O_2 as another possible mechanism in SIPS. Our results support this view. For example, PCNA was overexpressed in SIPS in hTERT-BJ1 cells. PCNA is involved in multiple functions, such as DNA repair, and some evidence suggests that it is regulated by p53 through $p21^{WAF-1}$ (Xu and Morris, 1999). The overexpression of PCNA is likely a part of the cellular response to DNA damage.

The two main features of HDFs in SIPS are that cells remain alive despite exposure to stress (they do not apoptose) and their cycle is irreversibly blocked. In normal conditions, a balance exists between the pro- and anti-proliferative signals and the pro- and anti-apoptotic signals. It could be that, in SIPS, this particular multi-variable balance gets reorganized, still allowing cell survival but blocking the cell cycle. The activation of pro-proliferative and anti-apoptotic signal transduction pathways could inhibit pro-apoptotic pathways while the activation of anti-proliferative pathways inhibit growth, e.g. $p21^{WAF-1}$ overexpression. For instance, p53 is known to regulate the expression level of $p21^{WAF-1}$ while the pro-apoptotic gene BAX is also known to be regulated by p53 (Schuler et al., 2003). On the other hand, p53 may regulate HIF-1, involved in the adaptation against oxidative stress. Indeed, activation of p53 by oxidative stress can result in either apoptosis or growth arrest and the events that determine the decision remain unclear (Martindale and Holbrook, 2002). Other examples exist, such as the overexpression of IGFBP5 or the downregulation of IGF1R. Of course that our results only reflect changes in a fraction of the transcriptome and so further studies are needed to confirm this hypothesis. Nonetheless, it is interesting to notice how both anti- and pro-apoptotic genes can be up- and downregulated as if to keep the balance between them (Fig. 2).

Another possibility is that oxidative stress directly activates pro-apoptotic pathways. Direct induction of signaling pathways by reactive oxygen species has been reported as part of the signaling cascade leading to growth arrest or apoptosis

Table 5
Genes differentially expressed in old, pre-senescent BJ HDFs when compared to BJ HDFs at early PDs

Symbol	Gene name	O/Y	Function	GenBank
Genes overexpressed in old BJ fibroblasts				
MMP3	Matrix metalloproteinase 3	↑ 35.4	Degradation of extracellular matrix	NM_002422
MMP1	Matrix metalloproteinase 1	↑ 6.5	Degradation of extracellular matrix	NM_002421
IL6	Interleukin 6	↑ 4.5	Immune response	NM000600
IGFBP5	Insulin growth factor binding protein5	↑ 4.4	Signal transduction	M65062
PLAU	Urokinase	↑ 3.6	Regulation of cell-surface plasminogen activation	NM_002658
PAI2	Plasminogen activator inhibitor type2	↑ 3.2	Fibrinolysis; cell cycle	J02685
PKM2	pyruvate-kinase-muscle 2	↑ 3.1	Energetic metabolism	M26252
ANX1	Annexin1	↑ 3.1	Anti-inflammatory	NM_000700
CTGF	Connective tissue growth factor	↑ 3.0	Cell proliferation	U14750
CAV1	Caveolin-1	↑ 2.9	Signal transduction; endocytosis; potocytosis	NM_001753
S100A	S100 calcium binding protein A4	↑ 2.7	Cell cycle control	NM_002961
p21	Cyclin dependent kinase inhibitor 1A	↑ 2.5	Cell cycle control	U03106
PCNA	Proliferating cell nuclear antigen	↑ 2.5	Control of DNA replication	NM002592
FGF2	Fibroblast growth factor 2	↑ 2.3	Cell proliferation	NM_002006
CDC42	Cell division cycle42	↑ 2.2	Cell cycle	NM_001791
AOP2	Anti-oxidant-protein2	↑ 1.7	Defense system	NM_004905
Genes not found expressed in young BJ fibroblasts				
EGFR	Epidermal growth factor receptor	↑	Control of cell growth and differentiation	NM_005228
CATB	Catenin, beta 1	↑	Cell adhesion; transduction signal	NM_001904
SMAD2	SMAD2	↑	Signal transduction	U68018
IL1B	Interleukin1 beta	↑	Inflammatory and immune responses	M15330
Genes underexpressed in old BJ fibroblasts				
FES	Feline sarcoma oncogene	↓ 2.9	Cell proliferation	X52192
HMOX	Heme-oxygenase	↓ 2.6	Defense system	NM_002133
CCNF	CyclinF	↓ 2.4	Cell cycle control	NM_001761
APOJ	ApolipoproteinJ	↓ 2.4	Lipid metabolism	J02908
SPRR1B	Cornifin	↓ 2.2	Cell structure	NM_003125
MYBL2	b-myb	↓ 2.1	Cell cycle control	X13293
IGF1R	Insulin like growth factor 1 receptor	↓ 2.1	Cell proliferation; anti-apoptotic	NM_000875
E2F2	E2F transcription factor2	↓ 2.1	Cell cycle control; apoptosis	NM_004091
CSF1R	Colony stimulating factor 1 receptor	↓ 2	Cell proliferation	NM_005211
BIN1	Bridging integrator 1	↓ 2	Cell cycle control; apoptosis	NM_004305
FGF8	Fibroblast growth factor 8	↓ 1.9	Cell proliferation	U36223
BAD	BCL2-antagonist of cell death	↓ 1.9	Pro-apoptotic	NM_004322
CASP3	Caspase3	↓ 1.9	Apoptosis	NM_004346
Gene not found expressed in old BJ fibroblasts				
ESR2	Estrogen receptor beta	↓	Cell–cell signalling	X99101

We calculated the ratio of the normalized hybridization intensity of old and young BJ cells (O/Y). Genes not expressed in one condition but whose value in the other condition is higher than the mean value for all genes in the corresponding array are represented as genes not found expressed.

(Macip et al., 2003). For example, maybe the increased DNA-binding activity of HIF-1 leads to the overexpression of FGF2 in H_2O_2-induced SIPS since FGF2 levels have been suggested to be mediated by HIF-1 (Li et al., 2002). It is possible that other signaling pathways are activated together with DNA-damage recognition. Since the stress employed was sublethal, cells did not apoptose and so the growth arrest prevailed. Yet at higher concentrations of H_2O_2, apoptotic pathways would likely prevail (Macip et al., 2003).

Previous reports indicated that hTERT transfection into BJ cells did not alter normal cellular functions (Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999); for instance, hTERT did not alter the karyotype. It was thus surprising to find so many differentially expressed genes between BJ and hTERT-BJ1 cells. Such abrupt differences indicate that the presence of telomerase does more than avoid the end-replication problem, as suggested by other recent results (Smith et al., 2003). This suggests that telomerase-immortalized cells, while not being transformed, do not have the same functional abilities of normal cells. In addition, recent results hint SOD2 as a tumor suppressor (Plymate et al., 2003). The observed downregulation of SOD2 in hTERT-BJ1 HDFs confirms reports that telomerase may favor tumorigenesis by a telomere length-independent mechanism (Stewart et al., 2002; Lindvall et al., 2003).

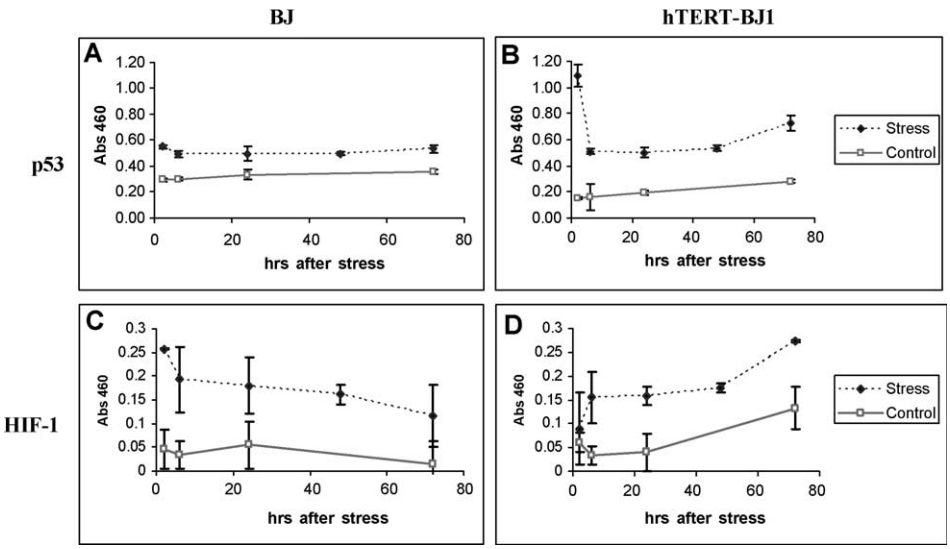

Fig. 3. Effect of a single H_2O_2 stress on the p53 (A and B) and HIF-1 (C and D) DNA-binding activity in BJ (A and C) and hTERT-BJ (B and D) cells. Samples were taken at 2, 6, 24, 48, and 72 h after stress.

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