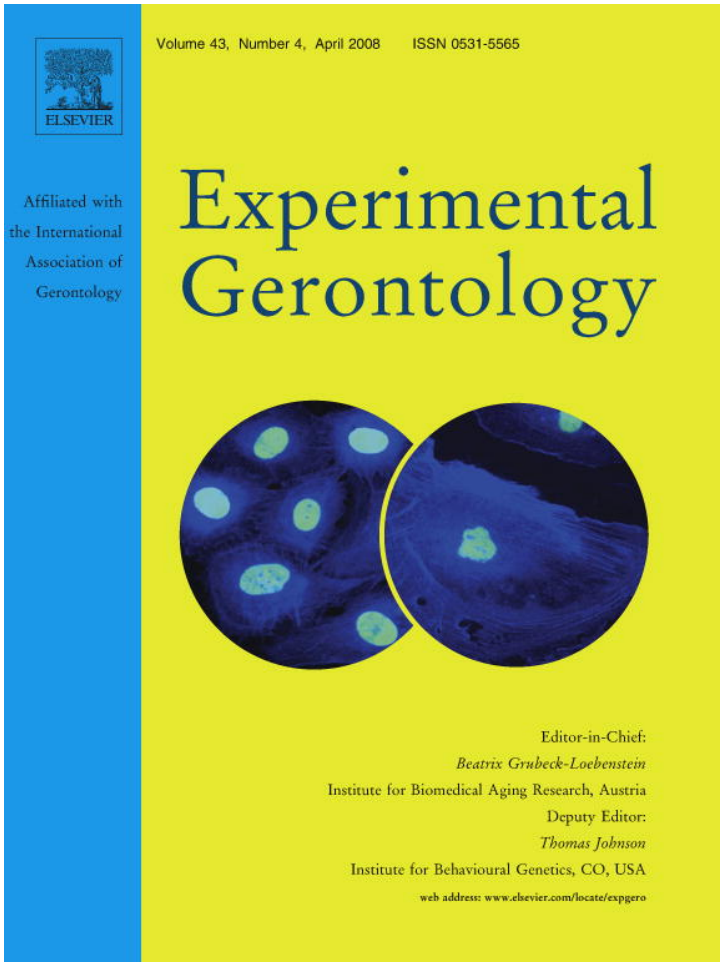

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Experimental Gerontology 43 (2008) 286–295
Experimental Gerontology
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Identification of replicative senescence-associated genes in human umbilical vein endothelial cells by an annealing control primer system

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Received 13 March 2007; received in revised form 14 December 2007; accepted 20 December 2007
Available online 10 January 2008

Abstract

Cellular senescence is regulated by specific genes in many organisms. The identification and functional analysis of senescence-associated genes could provide valuable insights into the senescence process. Here, we employed a new and improved differential display reverse transcription-polymerase chain reaction (DDRT-PCR) method that involves annealing control primers (ACPs) to identify genes that are differentially expressed in human umbilical endothelial cells during replicative senescence. Using 120 ACPs, we identified 31 differentially expressed genes (DEGs). Basic local alignment search tool (BLAST) search revealed 29 known genes and two unknown genes. Expression levels of the 29 known genes were confirmed by real-time quantitative RT-RCR and by Western blotting for eight of these genes. CD9 antigen, MHC class I chain-related sequence A (MICA) and cell division cycle 37 homolog (CDC37) were up-regulated, and bone morphogenetic protein 4 (BMP4), dickkopf-1 (DKK1), and transcription factor 7-like 1 (TCF7L1) were down-regulated in old cells. Treatment with recombinant human MICA caused a decrease in cell proliferation and an increase in senescence-associated β-galactosidase staining. Further analysis of differentially expressed genes may provide insights into the molecular basis of replicative senescence and vascular diseases associated with cellular senescence.

Keywords: Cell aging; Gene expression; Endothelial cells; DDRT-PCR

1. Introduction

Replicative senescence is the limited capacity of somatic cells to divide when cultured *in vitro* and is commonly studied as a model of biological aging (Hayflick and Moorhead, 1961). The phenotype of replicative senescence in human diploid fibroblasts (HDFs) is characterized by irreversible growth arrest in the transition from phase G1 to phase S of the cell cycle (Chen et al., 2000), larger and flattened cell morphology (Wagner et al., 2001a), expression of senescence-associated β-galactosidase (SA-β-gal) (Dimri et al., 1995), short telomeres (Deng et al., 2003; Harley et al., 1990), and altered gene expression (Cristofalo et al., 1998). Senescence occurs in a variety of cell types besides fibroblasts, including glial cells (Huang et al., 2006), keratinocytes (Kang et al., 2005), endothelial cells (Eman et al., 2006; Mueller et al., 1980), and is commonly accompanied by a specific set of changes in cell morphology, gene expression, and function. Using endothelial cells derived from human umbilical vein (HUVECs), *in vitro* senescence models have been described (Garfinkel et al., 1994; Wagner et al., 2001b) that might contribute to *in vivo* vascular cell aging and may thereby reveal pathomechanisms relevant to senescence-associated disorders of the human vascula-

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doi:10.1016/j.exger.2007.12.010

ture. Replicative senescence is associated with up- and down-regulation of a variety of genes involved in inflammation, cell cycle regulation, cytoskeleton, etc. (Yoon et al., 2004). Novel genes identified during cellular senescence have been analyzed by serial analysis of gene expression (SAGE) (Untergasser et al., 2002), differential display PCR (DD-PCR) (Linskens et al., 1995), and cDNA microarray technology (Yoon et al., 2004). Recently, an improved method to identify differentially expressed genes (DEGs) was developed that uses annealing control primers (ACPs) (Hwang et al., 2003; Kim et al., 2004).

In this study, to explore novel senescence-associated genes and to investigate their role in cellular senescence, we cultured HUVECs until they reached replicative senescence. We then identified DEGs associated with replicative senescence using ACPs. The expression levels of DEGs were validated by quantitative real-time RT-PCR and Western blot analysis. The possible roles for these genes in replicative senescence are discussed.

2. Materials and methods

2.1. Materials

HUVECs from three different donors and endothelial cell basal media supplemented with EGM singlequots were purchased from Cambrex BioScience, Inc. (Walkersville, MD). The AccuPrep gel purification kit and sequence-specific primers for senescence-associated genes (Table 1) were from Bioneer, Inc. (Daejeon, South Korea). The TOPO TA cloning kit was from Invitrogen, Inc. (Frederick, MD). The AccuPrep Plasmid Extraction kit was from Takara Biomedical, Inc. (Shiga, Japan). The LightCycler FastStart DNA Master SYBR Green I kit and LightCycler capillaries were from Roche, Inc. (Indianapolis, IN). Mouse monoclonal anti-BMP4 and anti-CDC37 antibodies and rabbit polyclonal anti-CD9 and anti-DKK1 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A rabbit polyclonal anti-TCF7L1 antibody and a mouse monoclonal anti-COTL1 antibody were from Abnova Corp. (Taipei, Taiwan). A goat polyclonal anti-MICA antibody and a mouse monoclonal anti-FZD4 antibody were from R&D Systems, Inc. (Minneapolis, MN). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Bio-Rad Laboratories, Inc. (Philadelphia, PA); anti-goat antibody was from Sigma–Aldrich, Inc. (St. Louis, MO). Recombinant human MHC class I polypeptide-related sequence A/Fc (MICA/Fc) chimera and control human IgG_1 Fc protein (Fc) were from R&D Systems, Inc. (Minneapolis, MN).

2.2. Cell culture and induction of senescence

HUVECs were cultured in endothelial cell basal medium supplemented with EGM singlequots and 10% FBS. Cells were harvested, propagated by trypsinization, and replated at 2×10^5 cells in 100-mm culture plates. When the subcultures reached 80–90% confluence, serial passaging was done by trypsinization. Population doublings (PD) were estimated using the following equation: $PD = (\log_{10} F - \log_{10} I)/\log_{10} 2$ (where F = number of cells at the end of one passage and I = number of cells at the beginning of one passage). After roughly 46 population doublings, cells reached growth arrest. For the experiments, cells were used in either passage 6 (PD < 16) or passage 15 (PD>52). These are referred to as 'young' and 'old' cells, respectively.

2.3. Senescence-associated β-galactosidase (SA-β-gal) staining

Senescent status was verified by *in situ* staining for SA-β-gal as previously described (Dimri et al., 1995). The percentage of blue cells per 400 cells observed under a light microscope was calculated.

2.4. Flow cytometry and cell cycle analysis

Cells were harvested, washed twice with PBS, and fixed with 70% ethanol at −20 °C for 1 h. After washing cells with PBS containing 2% FBS and 0.01% $CaCl_2$, RNase (1% w/v), and propidium iodide (50 μg/ml) were added and incubated at 37 °C for 30 min. The intracellular propidium iodide fluorescence intensity of 10,000 cells minimum was measured in each sample using a Becton–Dickinson FACS Caliber flow cytometer, and the cell cycle was analyzed by Cell Quest software (Becton–Dickinson, Inc., San Jose, CA).

2.5. Total RNA extraction and analysis of DEGs using ACPs

Total RNA was extracted from cells using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's suggestion. Total RNAs from young and old cells were entrusted to Seegene, Inc. (Seoul, South Korea) to identify DEGs by modified DDRT-PCR using ACPs (GeneFishing™ technology).

2.6. Cloning and sequencing of DEGs

Differentially expressed DNA fragments were separated in agarose gels, extracted from the agarose gels, and cloned using the TOPO TA cloning kit (Invitrogen, Inc., Frederick, MD). Following transformation of plasmids containing DEGs into *Escherichia coli*, plasmid DNAs were purified using the AccuPrep plasmid extraction kit. Sequences of DEGs were confirmed by dideoxy sequencing in Macrogen Co. (Seoul, South Korea). Identified DEGs were confirmed by BLAST or BLAT (BLAST-like alignment tool) searches (http://genome.ucsc.edu).

2.7. Real-time quantitative PCR

To confirm the DEGs identified from ACPs, real-time quantitative PCR was performed using a LightCycler 1.5

Table 1
Sequence-specific primers for real-time quantitative PCR of DEGs

DEG No.	Gene name		Primer sequence[a]	Length (bp)
1	CD9 antigen (CD9)	F	CATCTGCCCCAAGAAGGA	172
		R	GCGGATAGCACAGCACAA	
2	Dickkopf-1 (DKK1)	F	ACCCAGGCTCTGCAGTCA	182
		R	CCTGCAGGCGAGACAGAT	
3	Trafficking protein particle complex 4 (TRAPPC4)	F	GGGAAGCTCAGGCATTGA	248
		R	CCTTCTCTGCCACCTCCA	
4	Bone morphogenetic protein 4 (BMP4)	F	CATGCGGGATCTTTACCG	200
		R	CATGCGGGATCTTTACCG	
5	MHC class I polypeptide-related sequence A (MICA)	F	AACACCCAGTTGGGACGA	235
		R	TTGCAGCCTCCAACAACA	
10	FERM domain containing 4A (FRMD4A)	F	GGGAAGGGGAGGTTGAGA	292
		R	CCTTTACCGTGGCATTGG	
11	S100 calcium binding protein A6 (calcyclin) (S100A6)	F	GGGTAAACCGCGAATGTG	255
		R	GTGGAAGATGGCCACGAG	
13	Polymerase, δ2, regulatory subunit 50 kDa (POLD4)	F	CGTCTCCCGGGTTATCCT	299
		R	CAATGGTGGCCTGGTAGG	
14	Hypothetical protein LOC550643 (HPL550643)	F	TCCATTTCCCTGGTGCAT	271
		R	TGCTGCAGGACCATCTCA	
16	Utrophin (UTRN)	F	GTGCTGCCCTGCAAAACT	289
		R	ACCTGGAGGTTGGCATCA	
17	Coactosin-like 1 (*Dictyostelium*) (COTL1)	F	CATCACGTGGATCGGTGA	289
		R	TGGGCTGGTGGGCTAGTA	
18	Microtubule associated serine/threonine kinase 2 (MAST2)	F	GGGCTATGGGAAGCCAGT	290
		R	TCTGGCGGAGAAGTCCTG	
19	Chromosome 18 open reading frame 4 (C18orf4)	F	TACTCCGAGGCTGGTTGC	279
		R	ACGTCCAGCTTCCACTGC	
20	Metallothionein 1E (MT1E)	F	CCCTTTGCTCGAAATGGA	300
		R	GGGTTTGTGTCCCACGAG	
24	Zinc finger protein 292 (ZNF292)	F	GAGAGCAGGCCTTCACCA	226
		R	TCTTCGTCCGCCATCTTC	
25	Chaperonin containing TCP1, subunit 3γ (CCT3)	F	AGAACCCTCGCATTGTGC	245
		R	GACTCTGCGGATGGCTGT	
26	*Homo sapiens*, clone IMAGE:4414697	F	CCAAGACCTTCCGCTGAC	234
		R	GCCTGCAGTCCCAGCTAA	
28	Frizzled homolog 4 (*Drosophila*) (FZD4)	F	GCCAGAACCTCGGCTACA	234
		R	CGGGTTCACAGCGTCTCT	
31	Ornithine decarboxylase 1 (ODC1)	F	CCTTCGTGCAGGCAATCT	297
		R	CGTCATCAGAGCCCGTCT	
32	PCTAIRE protein kinase 1 (PCTK1)	F	CCTCGGATGAGGTGCAGT	260
		R	GGTACCCTCGCCCAGTTT	
33-1	Glycyl-tRNA synthetase (GARS)	F	CTTCGGCCTGGGTAGGAT	232
		R	TCCGATTGACCCAGAGGA	
33-2	*N*-sulfoglucosamine sulfohydrolase (SGSH)	F	GGTCCCACCGACACTCAC	201
		R	GCCCAGAAACACCACAGG	
33-3	Bone marrow stromal cell antigen 2 (BST2)	F	GAGTGCCCATGGAAGACG	279
		R	CCAGGGAAGCCATTAGGG	
34	Transcription factor 7-like 1 (TCF7L1)	F	GGAGATGAGGGCCAAGGT	258
		R	TCTGCCTCCTGGACTTGC	
37	Glioma tumor suppressor Candidate region gene 2 (GLTSCR2)	F	AAAGCGATGCCGATTCTG	241
		R	TCCTTGGAGCCAGTGTCC	

(*continued on next page*)

Table 1 (*continued*)

DEG No.	Gene name		Primer sequence[a]	Length (bp)
38	Hypothetical protein MGC49942 (HPM49942)	F	TGGCCGACTCTTCTCCTG	218
		R	CACCTTCTTGGGCCCTTT	
47	Denticleless homolog (*Drosophila*) (RAMP)	F	AACTGGGTGACCCGAACA	274
		R	TACCAGCAAGGCAGCACA	
48	5′,3′-nucleotidase, cytosolic (NT5C)	F	GTGCGGGAGATGAACGAC	251
		R	TGGCAGCAGGTGAACAAG	
53	Cell division cycle 37 homolog (*S. cerevisiae*) (CDC37)	F	GCTTCCGGCAGTTCTTCA	283
		R	GTGGGGTCCATCTTGCTG	

[a] F, forward primer; R, reverse primer.

(Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's suggestion. Synthesis of double-stranded DNA during various PCR cycles was monitored using SYBR Green I (Roche Applied Science, Indianapolis, IN).

2.8. Cell treatment with recombinant human MHC class I polypeptide-related sequence A (MICA/Fc) or Fc protein

Young HUVECs were seeded in 60-mm dishes (2×10^5 cells) or 96-well plates (1000 cells/well) and incubated for 24 h in EGM-2 medium. Cells were treated with MICA/Fc or Fc (200 or 400 ng/ml) for 4 days. Cell proliferation was measured by MTT assay and cellular senescence was analyzed by SA-β-gal staining.

2.9. Western blot analysis

Proteins (30 μg) were separated on 10% or 12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were soaked in 5% non-fat skim milk in TTBS (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) for 30 min at room temperature. Primary antibodies against CD9, DKK1, BMP4, MICA, CDC37, TCF7L1, FZD4, and COTL1 were applied overnight at 4 °C, the membranes were washed three times with TTBS and then HRP-conjugated secondary antibodies were applied for 2 h. After washing three times with TTBS for 30 min, antigen–antibody complex was detected using the enhanced chemiluminescence detection system (Neuronex, Inc., Pohang, South Korea). A rabbit polyclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a protein-loading control.

2.10. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) assay

Cells were seeded on 96-well plates at a density of 1×10^3 cells/well. After treatment with MICA/Fc or Fc (200 or 400 ng/ml) for 4 days, cells were incubated with 1 mg/ml MTT solution for 2 h. The medium was aspirated, and the formazan product was dissolved with 100 μl dimethyl sulfoxide. Cell proliferation was assessed by measuring absorbance at 570 nm with a Bio-Rad microplate reader.

3. Results

3.1. Characterization of replicative senescence in HUVECs

HUVECs were serially passaged until cell proliferation ceased. At 50 ± 3 population doublings, cells displayed large and flattened morphology compared to young cells (Fig. 1A). The percentage of blue cells, indicating SA-β-gal activity, was higher in senescent cells (86%) than in young cells (8%) (Fig. 1A and B). The PD time was also increased in senescent cells (Fig. 1C). To investigate the cell cycle status of senescent HUVECs, cells were stained with propidium iodide and analyzed by flow cytometry. While cell populations in S and G2/M phase were decreased in senescent cells compared to young cells, populations in G1/G0 phase were increased in senescent HUVECs (Fig. 1D). Since the levels of a variety of senescence-associated proteins such as FOXO3a (Kyoung Kim et al., 2005), pFOXO3a (Hu et al., 2005), p53 (Rosso et al., 2006), and p21 (Jackson and Pereira-Smith, 2006) were reported in cells entering replicative senescence, the levels of these proteins were measured by Western blotting. As expected, the FOXO3a protein level was decreased in old cells and the protein levels of pFOXO3a, p53, and p21 were increased (Fig. 1E).

3.2. Identification of DEGs during replicative senescence of HUVECs

To explore DEGs during replicative senescence, RNAs extracted from young and senescent cells were subjected to RT-PCR using a combination of 120 arbitrary primers and two anchored oligo(dT) primers (dT-ACP1 and dT-ACP2). Among the 400 amplicons analyzed, 53 differentially expressed DNA bands were identified between young and old cells (Fig. 2). Among these 53 DNA bands, 31 DNA bands were purified from agarose gels and cloned into TOPO TA-cloning vectors. The clones were sequenced, and the sequence similarities and characterization of these DEGs are summarized in Table 2.

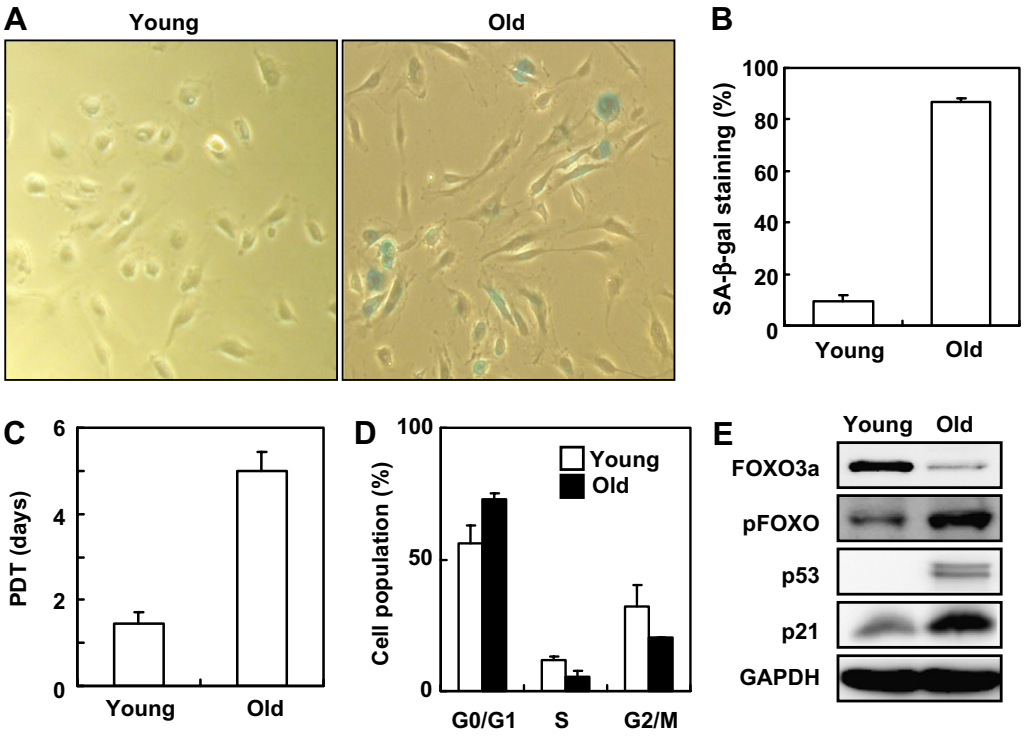

Fig. 1. Characterization of replicative senescence in HUVECs. (A) Morphological changes and SA-β-gal staining in young and old cells. (B) Percentages of SA-β-gal positive cells. (C) Measurement of population doubling times (PDT) in young and old cells. (D) Cell population in each phase of the cell cycle in young and old cells. (E) Western blot analysis of FOXO3a, pFOXO3a, p53, and p21 in young and old cells. Values are means ± SD from three independent experiments. Representative data from three independent experiments are shown.

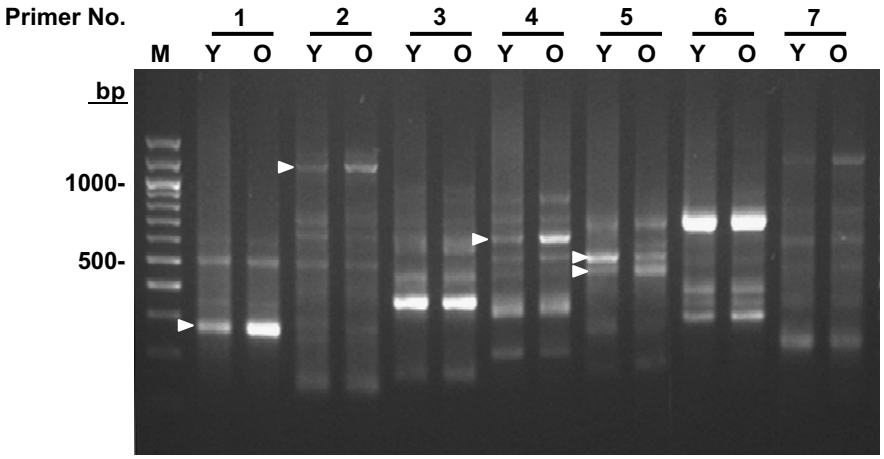

Fig. 2. Typical agarose gel electropherogram for PCR products amplified using ACPs between young (Y) and old (O) HUVECs. The amplified DNA products were separated on 1% agarose gels containing ethidium bromide. Arrowheads indicate the DEGs that show differential expression in young and old cells.

3.3. Validation of DEGs by real-time quantitative RT-PCR

To further determine the specific expression patterns of identified DEGs, 19 up-regulated genes and 10 down-regulated genes were selected and analyzed by fluorescence-monitored real-time RT-PCR. Real-time PCR showed that the expression levels of CD9 antigen (CD9), MHC class I polypeptide-related sequence A (MICA), frizzled homolog 4 (FZD4), coactosin-like 1 (COTL1), bone morphogenetic protein 4 (BMP4), zinc finger protein 292, transcription factor 7-like 1 (TCF7L1), and hypothetical protein MGC49942 were consistent with the initial expression patterns of DEGs (Table 2).

3.4. Protein expression analysis of selected candidate genes

To further evaluate the levels of several DEGs, whole cell lysates from both young and old cells were prepared

Table 2
Identification of DEGs associated with replicative senescence in HUVECs and validation of DEGs by real-time quantitative PCR

DEG No.	Gene name	DEG levels[a]	Real-time PCR (folds) [b]	Accession No.	Function
1	CD9 antigen	↑	2.257	NM_130850	Cell adhesion
2	Dickkopf-1	↑	NC	NM_012242	Development
5	MHC class I polypeptide-related sequence A	↑	2.028	NM_000247	Cellular defense
17	Coactosin-like 1 (*Dictyostelium*)	↑	1.804	NM_021149	Cytoskeleton
28	Frizzled homolog 4 (*Drosophila*)	↑	4.290	NM_012193	Wnt signaling
4	Bone morphogenetic protein 4	↓	0.162	NM_130850	Differentiation
16	Utrophin	↓	1.585	NM_007124	Cytoskeleton
24	Zinc finger protein 292	↓	0.331	XM_048070	Transcription
32	PCTAIRE protein kinase 1	↓	0.635	NM_006201	Cell cycle regulation
34	Transcription factor 7-like 1	↓	0.251	NM_031283	Transcription (Wnt signaling)
35	Biglycan	↓	0.298	NM_001711	Extracellular matrix
38	Hypothetical protein MGC49942	↓	0.478	NM_174893	Unknown
53	Cell division cycle 37 homolog (*S. cerevisiae*)	↓	1.907	NM_007065	Protein folding

[a] ↑, up-regulated in old cells; ↓, down-regulated in old cells.
[b] Values are means of three independent experiments; NC, not clear.

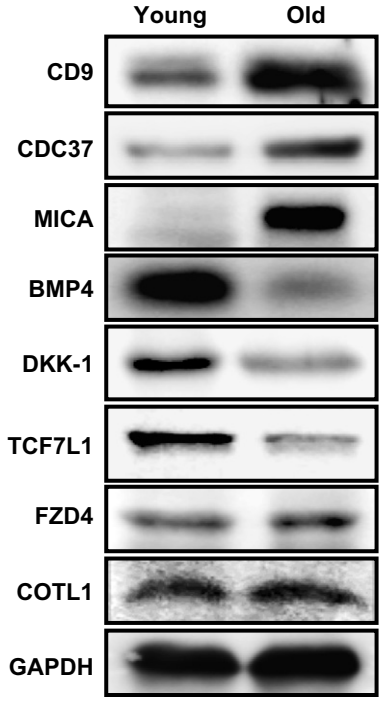

Fig. 3. Western blot analysis of DEGs in young and old HUVECs. Proteins were extracted from young and old cells and separated on SDS–polyacrylamide gels. The protein levels of CD9, CDC37, MICA, BMP4, DKK1, TCF7L1, FZD4, and COTL1 in young and old HUVECs were analyzed by Western blotting. Equal protein loading was estimated by using a GAPDH antibody. Representative data from three independent experiments are shown.

and the expression levels of candidate genes for which specific antibodies were available were analyzed by Western blotting. DKK1, BMP4, and TCF7L1 expression levels were decreased in old cells, and CD9, CDC37, and MICA protein levels were increased in senescent cells; these results were consistent with the DEG analysis and real-time PCR (Fig. 3). Although the FZD4 and COTL1 mRNA levels were increased in old cells, their protein levels were unchanged (Fig. 3).

3.5. Effect of MICA on cellular senescence in young HUVECs

To address whether up-regulation of MICA contribute to cellular senescence in HUVECs, we treated cells with MICA/Fc or control Fc protein and measured cell proliferation by MTT assay and SA-β-gal activity. Treatment with MICA/Fc caused a decrease in cell proliferation (Fig. 4A) and an increase in SA-β-gal staining (Fig. 4B). These results suggest that MICA might play an important role in the regulation of cellular senescence in HUVECs.

4. Discussion

A modified system of DDRT-PCR using ACP was used to detect genes that are differentially expressed during replicative senescence of HUVECs. ACP comprises a tripartite structure with a polydeoxyinosine linker between the 3′-end target core sequence and the 5′-end non-target universal sequence, which improves the specificity of PCR amplification and is therefore useful for the identification of differentially expressed genes (Hwang et al., 2003). From analysis of the expression levels of mRNA fragments, we found 53 DNA bands that were differentially expressed during replicative senescence of HUVECs. Among these 53 DEGs, 31 DEGs were selected and their expression patterns were confirmed by real-time RT-PCR. Real-time RT-PCR results showed that the expression levels of 4 of 19 (21%) up-regulated genes and 6 of 10 (60%) down-regulated genes were consistent with DEG analysis, which suggests that the DEG data require validation. We found that the protein levels of CD9, MICA, and CDC37 were up-regulated while the levels of DKK1, BMP4, and TCF7L1 protein were down-regulated in old cells. cDNA array technology (Hampel et al., 2006), subtractive hybridization (Grillari

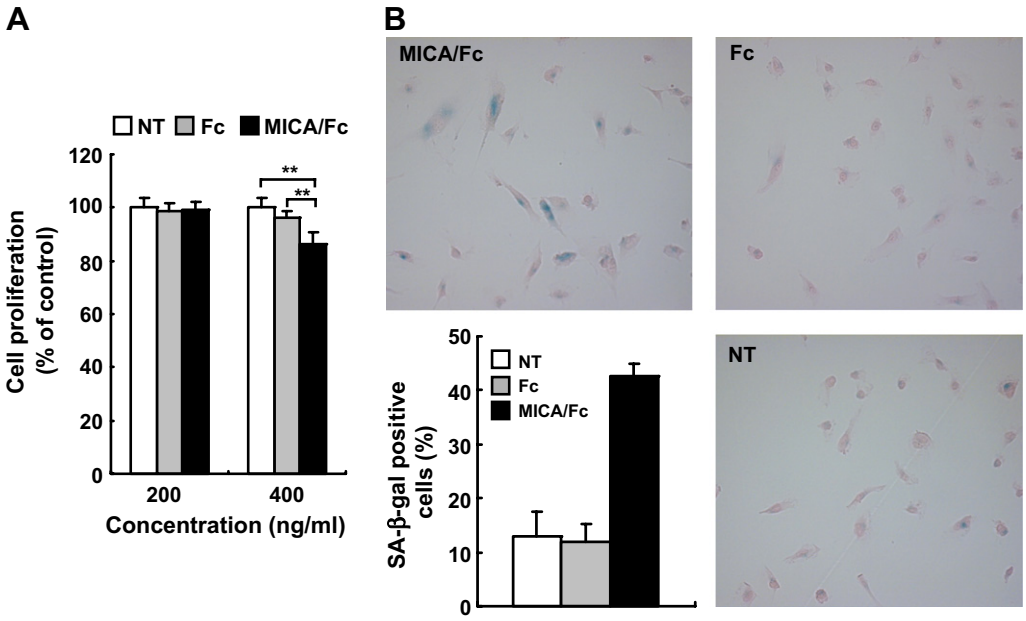

Fig. 4. Effects of MICA on cellular senescence. (A) Measurement of cell proliferation. Cells were treated with MICA/Fc or Fc (200 or 400 ng/ml) for 4 days and measured cell proliferation by MTT assay. Values are means ± SD of triplicates of four independent experiments and statistical significance was determined using the Student's *t* test (** $p < 0.01$). (B) SA-β-gal staining. Cells were treated with MICA/Fc or Fc (400 ng/ml) for 4 days and cellular senescence was observed by SA-β-gal staining. Values are means ± SD of three independent experiments. NT, not treated.

et al., 2000), and proteomic analysis (Bruneel et al., 2003) have been used to identify senescence-associated genes in HUVECs. In those experiments, up- and down-regulation were observed for a variety of genes, including insulin-like growth factor binding protein-3, insulin-like growth factor binding protein-5, interleukin-8, vascular endothelial growth inhibitor, TGF-β-inducible gene H3, p53-inducible gene (PIG3, a protein involved in vesicular transport), and ribosomal protein L28. DKK1 was reported to be up-regulated in senescent human dermal fibroblasts (Yoon et al., 2004). However, CD9, CDC37, MICA, BMP4, and TCF7L1 were not reported as differentially expressed in replicative senescence of HUVECs in other experiments. Therefore, this modified DDRT-PCR using ACPs might be a very good method for identifying novel DEGs.

CD9 antigen is a member of the transmembrane 4 (tetraspanin) superfamily that is known to complex with integrins and other transmembrane 4 superfamily proteins (Lanza et al., 1991). It can modulate cell adhesion and migration and also trigger platelet activation and aggregation (Lanza et al., 1991; Masellis-Smith and Shaw, 1994). CD9-deficient mice revealed only infertility of females due to a defect in the sperm–egg fusion process (Le Naour et al., 2000; Miyado et al., 2000). CD9 participates in endothelial cell migration during *in vitro* wound repair (Klein-Soyer et al., 2000), and monoclonal antibody against CD9 is reported to inhibit platelet-induced human endothelial cell proliferation (Ko et al., 2006), suggesting that CD9 plays an important role in endothelial regeneration. Although there were no reports of CD9 association with replicative senescence in endothelial cells, our finding that CD9 mRNA and protein levels were increased in old cells suggests that CD9 may play a role in cellular senescence of HUVECs.

Cell division cycle 37 (CDC37) was originally identified in yeast as a cell cycle mutant that gives a G1 arrest phenotype (Reed, 1980). CDC37 is bound to cyclin-dependent kinase-4 (CDK4) (Dai et al., 1996) and activates progression through G1 phase of the mammalian cell cycle by stabilizing CDK4 (Stepanova et al., 1996). As a key heat shock protein 90 (Hsp90) co-chaperone (Roe et al., 2004), CDC37 functions as a scaffold and facilitates binding of a variety of protein kinases to Hsp90, including Raf-1, Src family kinases, IKKs, and Akt/PKB (Pearl, 2005). Akt has been identified as one of the protein kinases associated with Hsp90 and CDC37 in a complex in which Akt is stabilized and regulated by phosphatidylinositol 3-kinase (Basso et al., 2002). In general, Akt activation has been reported to promote proliferation and survival of mammalian cells (Liang and Slingerland, 2003). However, Akt activity increased along with cellular senescence and Akt activation accelerated cellular senescence in primary human endothelial cells via the inactivation of FOXO3a and the p53/p21-dependent pathway (Miyauchi et al., 2004). Since cells in replicative senescence showed G1 arrest in the cell cycle, CDC37 might participate in replicative senescence of HUVECs by deregulating the cell cycle via CDK4 or Akt activation.

Major histocompatibility class I-related chain A (MICA) is a transmembrane glycoprotein that is found in many cell lines such as endothelial cells and fibroblasts (Zwirner et al., 1998). In fibroblasts and endothelial cells, MICA is induced upon heat shock and oxidative stress, and is also up-regulated in various tumors (Vivier et al.,

2002). Stress-inducible MICA enhances the function of NK and T cells by binding the stimulating receptor NKG2D (Bauer et al., 1999), and the activation of NKG2D receptor appears to play an important role in host defense against tumor formation (Smyth et al., 2005). Endothelial cells play important roles in inflammation and immune responses by regulating the expression levels in chemokines, adhesion molecules, and major histocompatibility complex II molecules as well as by modulating functions of natural killer cells and T cells (Methe et al., 2007). Therefore, the finding that treatment with recombinant MICA caused an increase in SA-β-gal staining suggests that MICA may play a role in endothelial cell senescence and contribute to immunological functions of endothelial cells.

In the present study, we showed that the levels of BMP4 RNA and protein decreased in senescent cells. Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily (Massague et al., 2000). BMP4 inhibits aortic and pulmonary vascular smooth muscle cell proliferation (Ma et al., 2001). There is also evidence that TGF family members can stimulate cell proliferation (Ohga et al., 1996). BMP4 phosphorylates p38 MAPK, ERK1/2, JNK, and Smad1 (Jeffery et al., 2005), and also activates NADPH oxidase, which leads to ROS production, NF-kB activation, intercellular adhesion molecule 1 expression, and subsequent increase the monocyte adhesivity of endothelial cells (Jo et al., 2006). BMP4 functions as a novel mediator of endothelial dysfunction and hypertension (Miriyala et al., 2006). To our knowledge, there were no reports of a role for BMP4 in cellular senescence of HUVECs. Hence, it is important to elucidate the relationship between BMP4 and replicative senescence in HUVECs.

TCF7L1 (transcription factor 7-like 1) is a member of the T cell factor/lymphoid enhancer factor (Tcf/Lef) family of transcription factors, which contain the high mobility group (HMG)-box DNA binding domain (Castrop et al., 1992). Tcf/Lef family transcription factors are the downstream effectors of the Wnt signal transduction pathway and are activated by β-catenin to stimulate transcription of a variety of target genes (Esufali and Bapat, 2004). A variety of Wnt receptors and transcriptional effectors are expressed in primary human endothelial cells, and the activation of Wnt/β-catenin signaling promotes the proliferation of endothelial cells as well as angiogenesis (Masckauchan et al., 2005). Wnt/β-catenin is also reported to communicate with p53 tumor suppressor (Harris and Levine, 2005), a common major effecter of cellular senescence in normal somatic cells (Ferbeyre et al., 2002). TCF7L1 levels were strongly increased in invading trophoblasts, and Wnt3A also stimulated trophoblast migration and invasion via TCF7L1 activation (Pollheimer et al., 2006). Wnt/β-catenin/TCF signaling plays a major role in the regulation of VSMC proliferation in response to growth factor stimulation by regulating the cell cycle genes cyclin D1 and p21 (Quasnichka et al., 2006). DKK1 (dickkopf-1) is a secreted protein with two cysteine-rich regions and is involved in embryonic development through its inhibition of the Wnt signaling pathway (Niehrs, 2006). Our finding that the expression levels of TCF7L1 and DKK1 are decreased in senescent HUVECs suggests the possibility that TCF7L1 and DKK1 contribute to replicative senescence by regulating cell proliferation via the Wnt signaling pathway.

In conclusion, our results demonstrate that gene expression is obviously changed in HUVECs during replicative senescence. Classification of the identified genes indicates that a comprehensive range of biological processes are involved in cellular aging. Further studies are needed to investigate the biological functions of these DEGs in replicative senescence of HUVECs, as well as in vascular diseases associated with senescence.

Acknowledgments

This work was supported by the Ministry of Science & Technology (MOST) and the Korea Science and Engineering Foundation (KOSEF) through the Aging-associated Vascular Disease Research Center at Yeungnam University (R13-2005-005-01003-0 (2007)) and by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ10-PG6-AG01-0003).

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