Microarray analysis of early and late passage chicken embryo fibroblast cells[1]

Byung-Whi Kong,*[2] Jeongyoon Lee,* Walter G. Bottje,* Kentu Lassiter,* Jonghyuk Lee,‡ Lauren E. Gentles,† Yohanna G. Chandra,* and Douglas N. Foster§

*Department of Poultry Science, Center of Excellence for Poultry Science, and †Department of Biological Sciences, University of Arkansas, Fayetteville 72701; ‡Department of Chemistry, Purdue University, West Lafayette, IN 47907; and §Department of Animal Science, University of Minnesota, St. Paul 55108

ABSTRACT Primary cultured cells derived from normal tissue have a limited lifespan due to replicative senescence and show distinct phenotypes such as irreversible cell cycle arrest and enlarged morphology. Studying senescence-associated genetic alterations in chicken cells will provide valuable knowledge of cellular growth characteristics, when compared with normal and rapidly growing cell lines. Microarray analysis of early- and late-passage (passage 4 and 18, respectively) primary chicken embryo fibroblast (CEF) cells was performed with a 4X44K chicken oligo microarray. A total of 1,888 differentially expressed genes were identified with a 2-fold level cutoff that included 272 upregulated and 1,616 downregulated genes in late-passage senescent CEF cells. Bioinformatic analyses were performed using Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com). Of the 1,888 differentially expressed genes in senescent CEF cells, 458 were identified as functionally known genes and only 61 genes showed upregulation. Because senescent cells generally showed the deactivated states of most cellular mechanisms for proliferation and energy metabolism, intensified analysis on upregulated genes revealed that the molecular mechanisms in senescent CEF cells are characterized by the suppression of cell cycle and proliferation, progression of cell death including apoptosis, and increased expression of various secreting factors. These regulatory pathways may be opposite to those found in the immortal CEF cell line, such as the DF-1 immortal line. Further comparison of differentially expressed genes between senescent and immortal DF-1 CEF cells showed that 35 genes overlapped and were oppositely regulated. The global gene expression profiles may provide insight into the cellular mechanisms that regulate cellular senescence and immortalization of CEF cells.

Key words: senescence, chicken embryo fibroblast cell, microarray

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INTRODUCTION

Replicative senescence is known to be an intrinsic mechanism in determining the finite lifespan of normal cells cultured in vitro (Hayflick and Moorhead, 1961). This process is recognized as an evolutionarily conserved mechanism in eukaryotic cells. Senescent cells display distinct phenotypic characteristics, such as growth arrest, enlarged cell morphology, cell cycle arrest, and telomere shortening (reviewed in Rodier and Campisi, 2011). A variety of genetic and epigenetic changes that include irreversible cell cycle arrest in replicative senescence, such as inactivation of p53, overexpression of p16INK4, and telomere regulatory functions, have been well characterized in human and mouse fibroblast cells (Sherr and DePinho, 2000). Most cells are unable to overcome senescence unless key tumor suppressor pathways are first altered. Thus, cellular senescence has been known to be a critical anticancer mechanism that suppresses cell growth at the risk for cellular transformation (Campisi, 2001).

Traditionally, primary chicken embryo fibroblasts (**CEF**) have been used for vaccine production, although primary CEF cells have the major disadvantage of inconsistent production of virus titer. Continuously growing or immortal avian cell lines that are not the result of infection with oncogenic viruses or treatment with carcinogenic chemicals but were established spontaneously provide a stable supply of identical cells for vaccine production. Several spontaneously immortalized CEF cell lines, such as the DF-1, SC-1, and SC-2 lines, have been established that may provide a consistent cellular source of poultry infectious viruses (Himly

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[2]Corresponding author: bkong@uark.edu

et al., 1998; Christman et al., 2005, 2006). Interestingly, although most CEF cells entered a senescent state after limited cell divisions, a small proportion of the cells regained their replication capacity and finally became immortalized. Theses observations suggest that the accumulation of genetic alterations may allow a subpopulation of CEF cells to overcome cellular senescence leading to immortalization. Although the alteration of p53, p16INK4, and telomere regulatory functions during replicative senescence were demonstrated previously in primary CEF cells (Kim et al., 2002), the genome-wide transcriptional alterations of senescent CEF cells have yet to be determined.

Previously, we reported the global gene expression profiles in spontaneously immortalized DF-1 CEF cells showing enhanced proliferation rates, hypersensitivity to oxidative stress, and hyperactive mitochondrial functions (Kim et al., 2001; You et al., 2004; Kong et al., 2011). The microarray data revealed that immortal DF-1 CEF cells were characterized by enhanced molecular mechanisms for cell cycle progression and proliferation, suppression of apoptosis, altered cellular morphogenesis, and accelerated capacity for molecule transport. The immortal DF-1 cell line was derived from CEF cells initially isolated from *ev-0* (endogenous virus free) embryos (Himly et al., 1998). Moreover, the DF-1 cell genome is currently being sequenced (personal communication, Jerry Dodgson, Michigan State University, East Lansing). Therefore, the major objective of the present study was to conduct defined epigenetic studies with primary nonsenescent (young), senescent, and DF-1 immortal CEF cells to specifically investigate critical factors involved in modulation of cell proliferation and lifespan. In this study, genome-wide differential gene expression in senescent CEF cells was determined using microarray analysis and was further compared (contrasted) with immortal DF-1 cells to investigate potential genetic factors that could initiate the immortal phenotype in chicken cells.

MATERIALS AND METHODS

Cell Culture

All materials and methods including cell culture, RNA isolation, microarray, reverse-transcription PCR, and bioinformatics were followed as previously reported (Kong et al., 2011) with modifications. Briefly, cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA). Primary chicken CEF cells were isolated from 10-d-old specific-pathogen free (SPF) chicken embryos (Charles River Laboratories, North Franklin, CT). Single cell populations were obtained by 0.25% trypsin/1 m*M* EDTA treatment to dissociate cells. Primary CEF cells were suspended in Dulbecco's modified Eagle's medium (0.45% glucose) plus 10% fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 2 m*M* L-glutamine in 10-cm tissue culture dishes (Sarstedt Inc., Newton, NC). Cultured cells were grown at 39°C in a 5% CO_2 incubator until cells reached confluence (2 to 4 d) and primary CEF cells were passaged every 3 to 4 d for 2 mo until cells ceased growing. Cells collected at the fourth passage were designated as young CEF cells, whereas those collected at the eighteenth passage were designated as senescent CEF cells. All procedures for handling chicken embryos, cell culture, and DNA/RNA isolation were approved by the Institutional Biosafety Committee (IBC: protocol number: 10007) at the University of Arkansas.

Total RNA Extraction

Total RNA was extracted from young (passage 4) and senescence (passage 18) CEF cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's instructions. Total RNA was treated with DNase I (New England BioLabs Inc., Ipswich, MA), and RNA was repurified by TRIzol reagent.

Probe Labeling and Microarray Hybridization

The 4X44K Agilent chicken oligo microarray (array ID: 015068) and a 2-color labeling microarray system kit (Agilent Technologies, Palo Alto, CA) was used to compare mRNA expression between young- and senescent CEF cells. Fluorescently labeled complementary RNA (**cRNA**) probes were generated following the manufacturer's instructions. The RNA Spike-in controls were used to adjust possible dye effects following the manufacturer's instructions (Zahurak et al., 2007; Lee et al., 2010). Briefly, 2 μg of total RNA was converted to cDNA using reverse transcriptase and an oligo dT primer in which the T7 promoter sequence was added. T7 RNA polymerase was used for the synthesis and labeling of cRNA with either Cy3 dye for the young CEF control or Cy5 dye for senescent CEF RNA samples. The concentration, fluorescent intensities, and quality of labeled cRNA probes were determined using a Nano-drop spectrophotometer (Thermo Scientific, Wilmington, DE). After hybridization, slides were scanned using a Genepix 4000B scanner (Molecular Devices Corporation, Sunnyvale, CA) with the tolerance of saturation setting of 0.005%. Four biological replicates were conducted.

Microarray Data Collection and Analysis

Background-corrected red and green intensities were normalized by the local polynomial regression (LOWESS) method. Genes (array spots) of the 44K array with significant signal intensities were sorted by absolute real (foreground) fluorescent signal >100 and signal to noise ratio >3, meaning that real signals of the samples were 3 times greater than background signals.

Table 1. Primers used for quantitative reverse-transcription PCR

Accession no.	Forward primer (5′ → 3′) Reverse primer (5′ → 3′)	Gene symbol
BX931599	CTGTTTCCTGACCGCAGTTC AGCACAAACTCCGCCATTTT	*VIPR2*
BX933478	CCTGTGCAAGGTGTCCAGTG CCCAATGGCCATACAGTTCA	*MXRA5*
BX933888	CTGGGATCCCTCCAGAGCTA CCATTCACTGGAGCACCAAA	*C1QTNF3*
BX934511	TGGAACCTGGAACCTTGTCA TGTGCTTGTGGTATGGATGGA	*RBP7*
BX936211	CCAGCTGTCCTCCTTGGAAT AGGGAGAGGAAGACGTGCTG	*TMEM116*
CF252431	ACCAGGAAGATGGCTGGTGT TCTGTCATCTGCTGTGGTCTCA	*FABP4*
CR523499	GCAATGGACAGTGACCAACC AGCCCCTCCCTGTACAAAGC	*OSGIN1*
M80584	TCCCACTGAGCAGCTTCTGTA CCAGAGAGATATCCGCAGCA	*LUM*
M87294	GGTGCTGACTTTCGCCTTGT GCCTGGTGATGAGGTTGATG	*NPY*
X77960	CTGGGGTGTTGCCTCTTTTC TCCCGGGAGAATACAAAGGA	*RBP4*
X87609	CCACCTGAGAAAAGCGACCT ACATCGACCTCTGCCAACCT	*FST*
NM_204305	GGCACTGTCAAGGCTGAGAA TGCATCTGCCCATTTGATGT	*chGAPDH*

To identify differentially expressed genes, a moderated *t*-statistic and its corresponding *P*-value based on empirical Bayes methods (Smyth, 2004) for each gene, and false discovery rate (**FDR**) were computed using JMP Genomics tool (SAS Institute Inc., Cary, NC), which is licensed to the Cell and Molecular Biology Program at the University of Arkansas. The genes with both a FDR below 0.01 and fold change over ± 2 fold were considered as different between 2 groups and identified as differentially expressed genes. Results were deposited into Gene Expression Omnibus (**GEO**; accession number: GSE29258).

Quantitative Reverse-Transcription PCR

Reverse transcription was performed using 3 μg of total RNA, Superscript II reverse transcriptase and oligo $dT_{12–18}$ primers (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's instructions. The reverse-transcribed cDNA was diluted 1:10 and a portion (1 μL) was subjected to quantitative reverse-transcription PCR (**qPCR**) under the following conditions: 40 cycles of 95°C for 30 s, gene-specific annealing temperature (58 to 65°C) for 1 min, extension for 30 s at 72°C, and a final extension at 72°C for 10 min. A nontemplate control and endogenous loading control (chicken GAPDH) were used for the relative quantification. The differential expression in senescent CEF cells was calculated by the $-\Delta\Delta CT$ method, which is comparable with the $\log_2$ value of differentially expressed genes (Livak and Schmittgen, 2001), against the primary CEF counterpart. Primers for qPCR were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) and were synthesized by Integrated DNA Technologies (Coralville, IA). Primer information is listed in Table 1. All qPCR reactions were performed 3 times.

Bioinformatics

Functional interpretation of differentially expressed genes was analyzed in the context of gene ontology and molecular networks using the Ingenuity Pathways Analysis (IPA; Ingenuity Systems; http://www.ingenuity.com).

RESULTS AND DISCUSSION

Morphology and Growth Characteristics of Young and Senescent CEF Cells

Normal primary CEF cells can grow ~18 to 20 passages in culture dishes for approximately 2 mo. Passage 18 senescent CEF cells morphologically were enlarged in size and flattened and contained many vacuoles compared with primary passage 4 young CEF cells (Figure 1A and B). Growth rates of senescent CEF cells had declined to between 0.1 to 0.2 population doublings per day (**PD**/d) compared with 0.6 to 0.8 PD/d of primary young passage of CEF cell counterpart (Figure 1C).

Gene Expression Profile of Senescent CEF Cells

Genome-wide expression profiling was conducted using RNA from young and senescent CEF cell samples. Of the 44K genes used in the microarray analysis, a total of 1,888 differentially expressed genes were identified in senescent CEF cells with a 2-fold level cutoff that included 272 upregulated and 1,616 downregulated

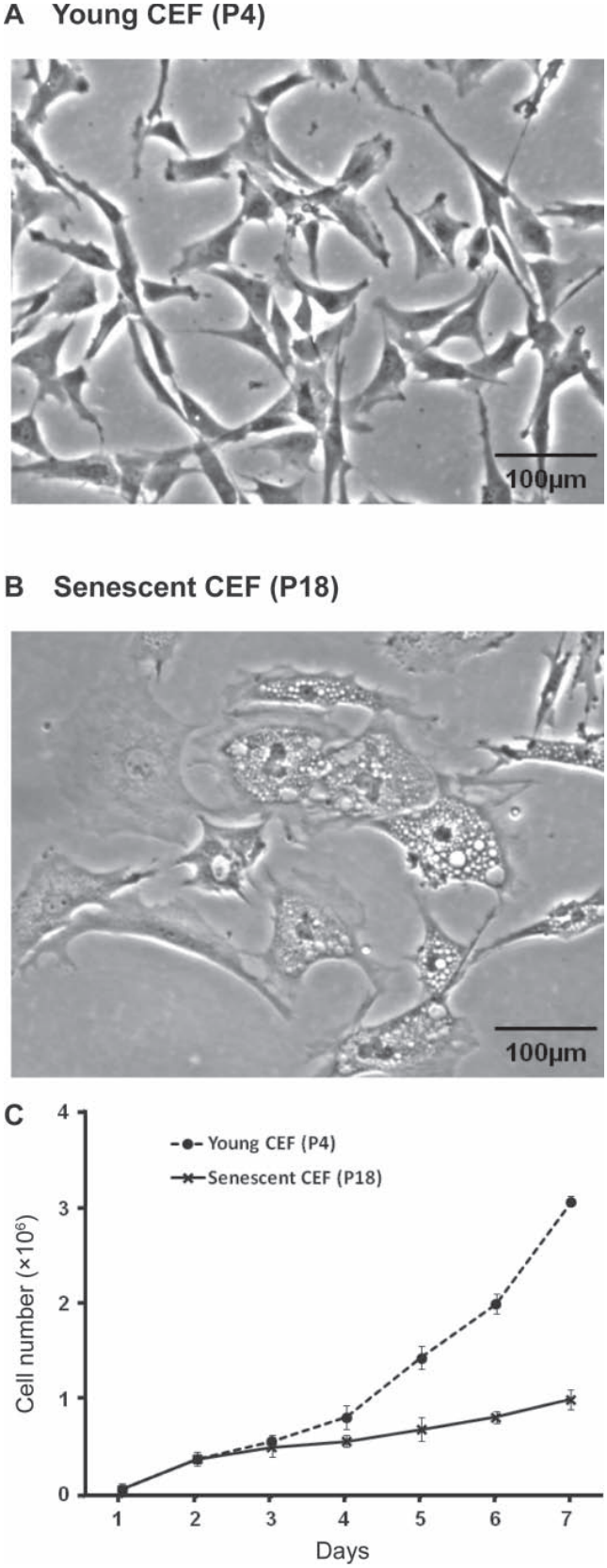

Figure 1. Morphology and cell growth kinetics for early passage (passage 4) young primary and late passage (passage 18) senescent chicken embryo fibroblast (CEF) cells. Cell images for young primary (P4) (A) and senescent (P18) CEF (B) cells were obtained by inverted microscopy. A scale bar is shown for each image. (C) Growth kinetics. Young and senescent CEF cells were seeded at 1×10^5 cells per 10-cm dish, and the accumulated cell numbers were counted at each day for 7 d. Experiments were repeated 3 times.

genes compared with young CEF cells (Supplemental File 1; available online at http://ps.fass.org/). To validate the microarray results, 11 randomly chosen genes from the list of the 1,888 differentially expressed genes were subjected to qPCR in addition to the GAPDH loading control gene. Results indicated that increased or decreased expression levels for all genes tested were well-matched in assays between microarray and qPCR analysis (Table 2). When the 1,888 differentially expressed genes were analyzed using Ingenuity Pathways Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com), 458 were classified as functionally known genes (Supplemental File 2; available online at http://ps.fass.org/). The majority of genes were downregulated in senescent CEF cells, whereas only 61 functionally known genes were upregulated compared with young CEF cells (indicated by red type in Supplemental File 2). This is not surprising because senescent cells are inactive in most cellular mechanisms for proliferation and energy metabolism pathways as tumor suppressor mechanisms (Campisi, 2001). Thus, the following discussion will focus on genes exhibiting the greatest degree of upregulation relative to expression in young (passage 4) cells. We hypothesize that the upregulated genes may be particularly important with regard to mechanisms involved in induction of cellular senescence in CEF cells.

Functional Groups of Upregulated Genes in Senescent CEF Cells

The IPA program generated bioinformatic data sets including functional groups (gene ontology; GO) and

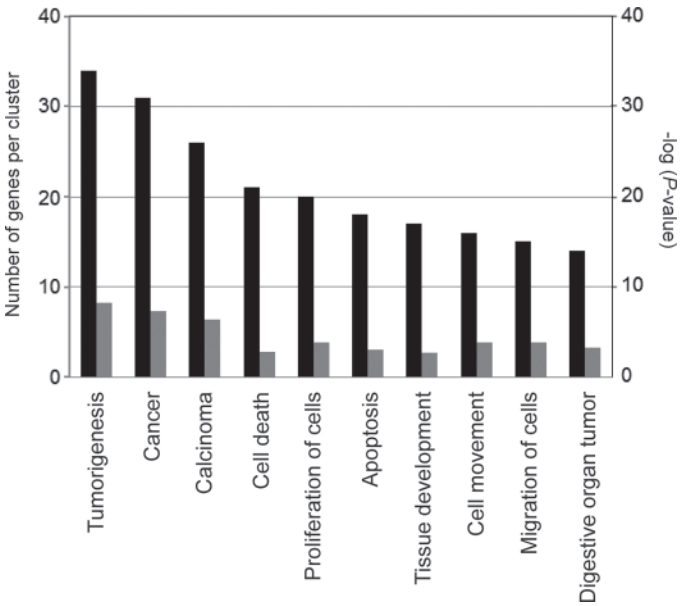

Figure 2. Functional gene ontology (GO) for upregulated genes. Genes (61) were categorized into functional groups by the Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com) program. Black bars represent the number of genes for each cluster, whereas gray bars indicate –log(*P*-value), which was calculated by IPA software showing the levels of relatedness.

Table 2. Comparison of fold changes between microarray and quantitative reverse-transcription PCR (qPCR)[1]

Accession no.	Gene symbol	Microarray	qPCR
BX931599	*VIPR2*	−2.5	−2.3
BX933478	*MXRA5*	−1.4	−1.1
BX933888	*C1QTNF3*	−4.9	−2.2
BX934511	*RBP7*	4.1	5.9
BX936211	*TMEM116*	1.2	1.7
CF252431	*FABP4*	6.3	8.1
CR523499	*OSGIN1*	3.4	3.6
M80584	*LUM*	−2.6	−1.0
M87294	*NPY*	2.6	1.2
X77960	*RBP4*	3.6	1.9
X87609	*FST*	2.1	1.1

[1]The gene expression levels of 11 genes from microarray analysis were confirmed by qPCR. The expression levels were presented by $\log_2$ fold change values in microarray analysis, whereas, for qPCR, the values were calculated by the ΔΔCT method, which were comparable with $\log_2$ fold changes in the microarray. All values are mean values determined by the calculations from 3 replicate assays.

gene networks for upregulated genes in senescent CEF cells. When the 61 genes showing upregulated expression were categorized functionally into biological groups (Figure 2), the greatest number of genes was mainly categorized into functionalities of tumorigenesis, cancer, carcinoma, cell death, proliferation, apoptosis, tissue development, cell movement, migration, and digestive organ tumor, suggesting that the transcriptional regulation that occurred in aged senescent CEF cells is closely related, and likely responsible, for the dramatic loss of proliferation capability and the potential induction of apoptosis.

The 10 Most Upregulated Genes in Senescent CEF Cells

The 10 most upregulated genes (Table 3 and Supplemental File 3; available online at http://ps.fass.org/) focus on the functions of cellular proliferation, stress, and apoptosis. Of these, the upregulation of *RBP4*, *OSGIN1*, and *FAH* is closely associated with the antiproliferative function and the induction of apoptosis. The *RBP7*, *AKR1B10*, and *NTS* are incongruous with the replicative senescence, but may contribute to the cellular transformation to tumor cells from senescent cells (Supplemental File 3). The *NPY* and *GSTT1* are known to play roles in cellular stress responses (Supplemental File 3), whereas the functions for *ATP1B1* and *ALDH1A3* in senescent CEF cells are unknown. Thus, further study is needed to reveal the functional roles of these genes in senescent CEF cells.

Gene Networks

Gene network analysis, which represents the intermolecular connections among interacting genes based on functional knowledge inputs, was performed on the upregulated genes in senescent CEF cells using the IPA program. Though the simplest setting (35 focus molecules and 10 networks) was employed to analyze molecular gene networks, only 3 networks were generated due to relatively small number (61) of genes in this subset. Networks with minor modification (to show clearer interactions) are displayed in Figures 3A, 3B, and 3C. The functional group of genes involved in each network is listed in Table 4, and gene information for focus molecules in each network is listed in Supplemental File 4 (available online at http://ps.fass.org/).

Network #1 centers on the NF-κB signaling pathway and the top functions for Network #1 are cellular movement, cellular growth/proliferation, and cell death (Figure 3A). Increased expression for ATP6AP2 [ATPase, H^+ transporting, lysosomal accessory protein 2; (pro)renin receptor (PRR)], ALDH1A3, LSP1

Table 3. The 10 most upregulated genes[1]

Symbol	ID	Entrez gene name	Log_2 ratio	*P*-value
RBP7	BX934511	Retinol binding protein 7, cellular	4.12	2.51×10^{-5}
RBP4	X77960	Retinol binding protein 4, plasma	3.58	7.62×10^{-5}
OSGIN1	CR523499	Oxidative stress-induced growth inhibitor 1	3.42	3.19×10^{-5}
ATP1B1	J02787	ATPase, Na^+/K^+ transporting, β 1	2.75	1.09×10^{-5}
NPY	M87294	Neuropeptide Y	2.61	5.22×10^{-4}
GSTT1	U13676	Glutathione S-transferase theta 1	2.18	3.50×10^{-6}
FAH	BX935011	Fumarylacetoacetate hydrolase	2.13	1.96×10^{-4}
AKR1B10	AJ295030	Aldo-keto reductase family 1, member B10	1.96	4.77×10^{-5}
NTS	BX931642	Neurotensin	1.92	2.33×10^{-4}
ALDH1A3	AF152358	Aldehyde dehydrogenase 1 family, A3	1.91	3.70×10^{-3}

[1]Fold change values were indicated by $\log_2$. All genes were matched and verified with UniGene function of the National Center for Biotechnology Information database.

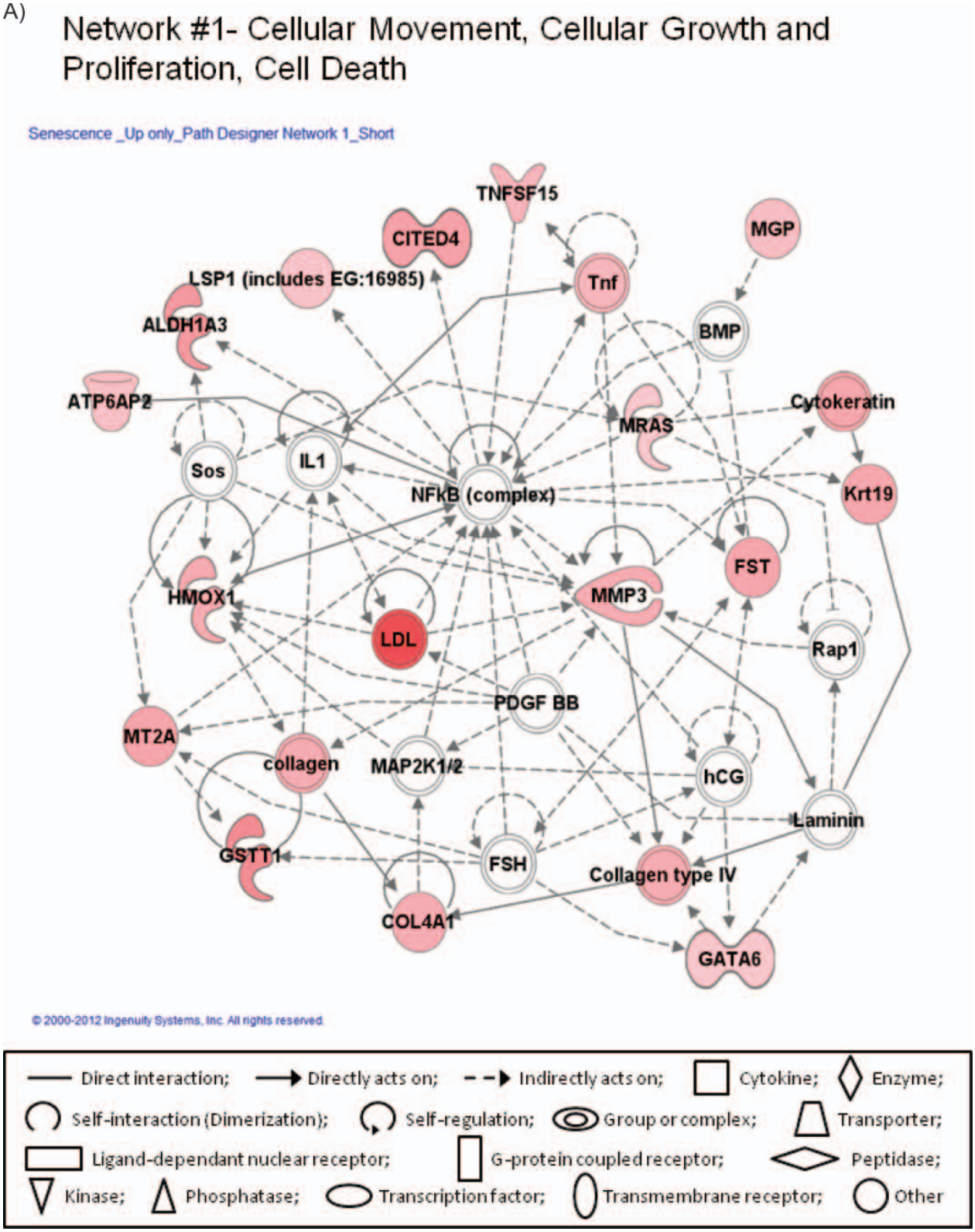

Figure 3A. Gene network analysis for upregulated genes. (A) Network #1, (B) Network #2, (C) Network #3. Molecular interactions among important focus molecules are displayed. Filled symbols depict upregulated genes, whereas white symbols indicate neighboring genes that are functionally associated but not included in the differentially expressed gene list. Symbols for each molecule are presented according to molecular functions and type of interactions. ALDH1A3, aldehyde dehydrogenase 1 family, member A3; ATP6AP2, ATPase H+ transporting lysosomal accessory protein 2; BMP, bone morphorenetic protein; CITED4, Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 4; COL4A1, collagen type IV alpha 1; FSH, follicle-stimulating hormone; FST, follistatin; GATA6, GATA binding protein 6; GSTT1, glutathione S-transferase theta 1; hCG, human chorionic gonadotropin; HMOX1, heme oxygenase (decycling) 1; Krt19, keratin 19; LDL, low-density lipoprotein; LSP1, lymphocyte-specific protein 1; MGP, matrix Gla protein; MMP3, matrix metallopeptidase 3 (stromelysin 1, progelatinase); MRAS, muscle RAS oncogene homolog; MT2A, metallothionein 2A; NFkB, nuclear factor kappa B; PDGF BB, platelet-derived growth factor BB; PLC, phospholipase C; Rap1, RAS oncogene family member 1; Sos, son of sevenless homolog 1; Tnf, tumor necrosis factor; TNFSF15, tumor necrosis factor (ligand) superfamily member 15. Color version available in the online PDF.

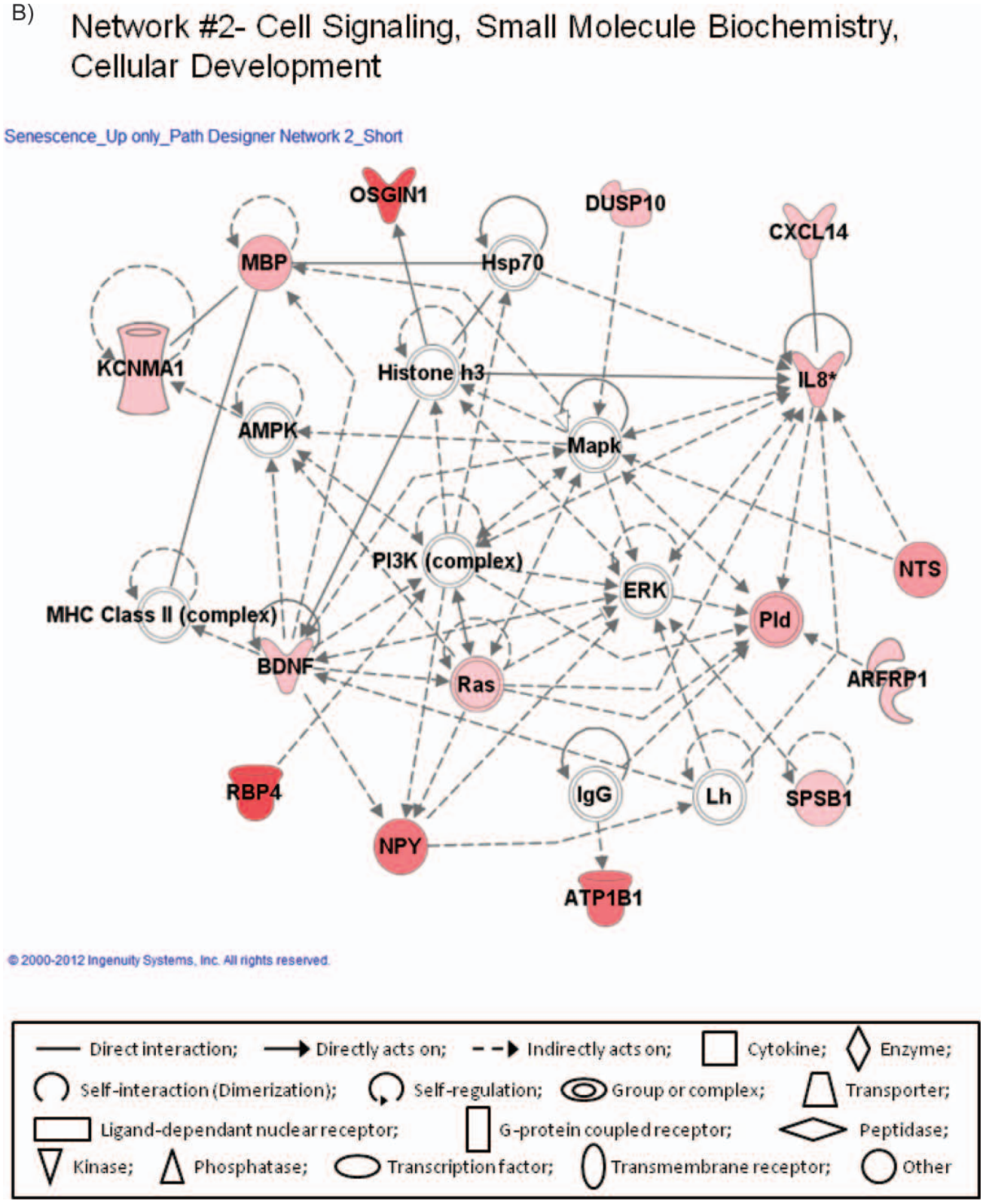

Figure 3B. Gene network analysis for upregulated genes. (A) Network #1, (B) Network #2, (C) Network #3. Molecular interactions among important focus molecules are displayed. Filled symbols depict upregulated genes, whereas white symbols indicate neighboring genes that are functionally associated but not included in the differentially expressed gene list. Symbols for each molecule are presented according to molecular functions and type of interactions. AMPK, AMP-activated protein kinase; ARFRP1, ADP-ribosylation factor-related protein 1; ATP1B1, ATPase Na+/K+ transporting beta 1 polypeptide; BDNF, brain-derived neurotrophic factor; CXCL14, chemokine (C-X-C motif) ligand 14; DUSP10, dual-specificity phosphatase 10; ERK, extracellular signal-regulated kinases (p42/p44 Mapk); Histone h3, histone subtype h3; Hsp70, heat shock protein 70 kDa; KCNMA1, potassium large conductance calcium-activated channel subfamily M alpha member 1; Lh, luteinizing hormone; Mapk, mitogen-activated protein kinase; MBP, myelin basic protein; MHC Class II (complex), major histocompatibility complex class II; NPY, neuropeptide Y; NTS, neurotensin; OSGIN1, oxidative stress induced growth inhibitor 1; PI3K (complex), phosphoinositide 3 kinase; Pld, phospholipase D; Ras, Ras oncogene; RBP4, retinol binding protein 4; SPSB1, SplA/ryanodine receptor domain and SOCS box containing 1. Color version available in the online PDF.

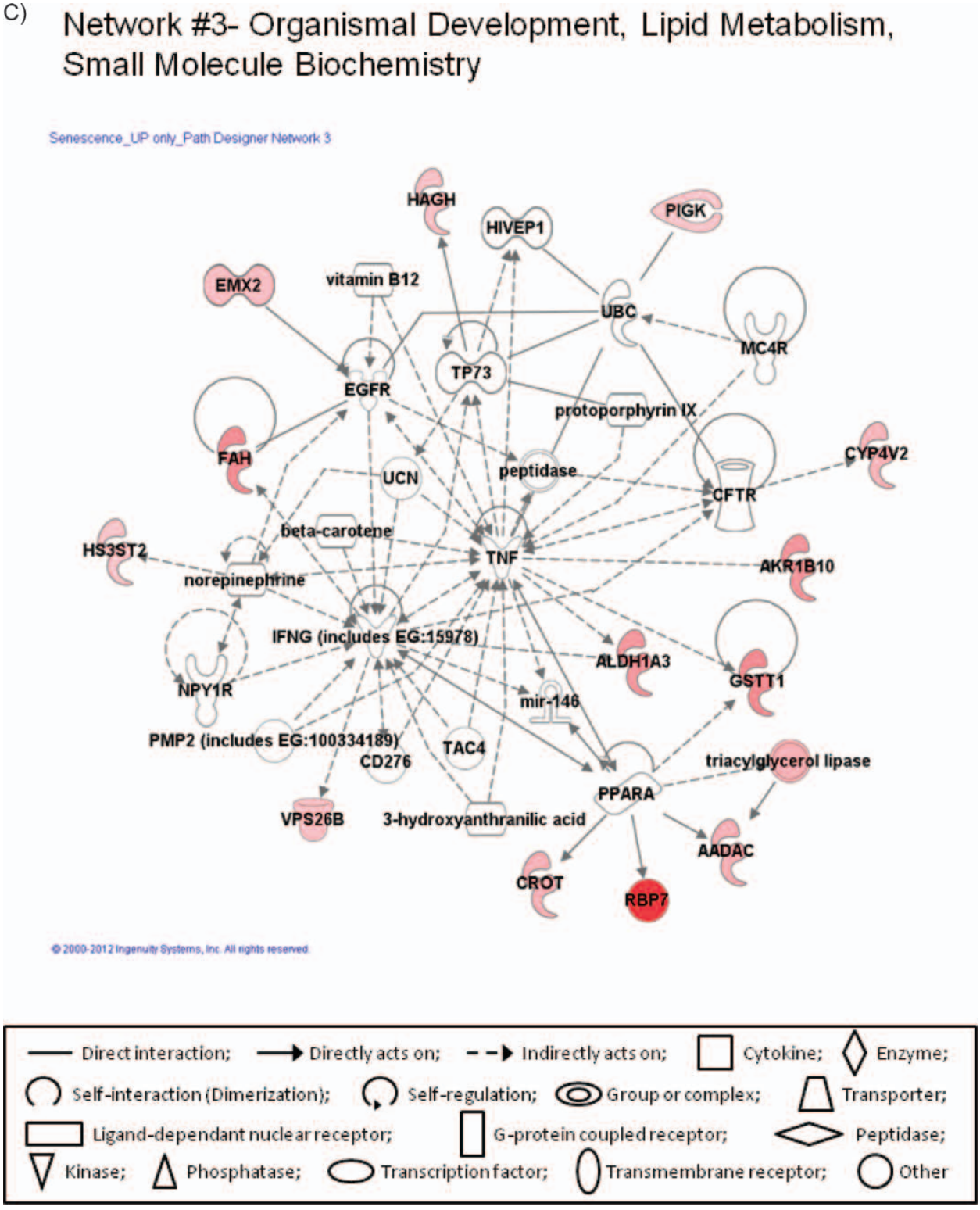

Figure 3C. Gene network analysis for upregulated genes. (A) Network #1, (B) Network #2, (C) Network #3. Molecular interactions among important focus molecules are displayed. Filled symbols depict upregulated genes, whereas white symbols indicate neighboring genes that are functionally associated but not included in the differentially expressed gene list. Symbols for each molecule are presented according to molecular functions and type of interactions. AADAC, arylacetamide deacetylase (esterase); AKR1B10, aldo-keto reductase family 1 member B10 (aldose reductase); ALDH1A3, aldehyde dehydrogenase 1 family member A3; CD276, CD276 molecule; CFTR, cystic fibrosis transmembrane conductance regulator (ATP-binding cassette subfamily C member 7); CROT, carnitine *O*-octanoyltransferase; CYP4V2, cytochrome P450 family 4 subfamily V polypeptide 2; EGFR, epidermal growth factor receptor; EMX2, empty spiracles homeobox 2; FAH, fumarylacetoacetate hydrolase (fumarylacetoacetase); GSTT1, glutathione S-transferase theta 1; HAGH, hydroxyacylglutathione hydrolase; HIVEP1, human immunodeficiency virus type I enhancer binding protein 1; HS3ST2, heparin sulfate (glucosamine) 3-*O*-sulfotransferase 2; IFNG (includes EG:15978), interferon gamma; MC4R, melanocortin 4 receptor; NPY1R, neuropeptide Y receptor Y1; PIGK, phosphatidylinositol glycan anchor biosynthesis class K; PMP2 (includes EG:100334189), peripheral myelin protein 2; PPARA, peroxisome proliferator-activated receptor alpha; RBP7, retinol binding protein 7; TAC4, tachykinin 4 (hemokinin); TNF, tumor necrosis factor; TP73, tumor protein p73; UBC, ubiquitin C; UCN, urocortin; ULBP2, UL16 binding protein 2; VPS26B, vacuolar protein sorting 26 homolog B (*Schizosaccharomyces pombe*). Color version available in the online PDF.

Table 4. Network functions for upregulated genes in senescent chicken embryo fibroblast cells[1]

ID	Associated network functions	Score	Focus molecules
1	Cellular movement, cellular growth and proliferation, cell death	31	15
2	Cell signaling, small molecule biochemistry, cellular development	30	14
3	Organismal development, lipid metabolism, small molecule biochemistry	25	13

[1]Functions associated with 3 networks are listed. Score means the number of network eligible molecules out of differentially expressed genes.

(lymphocyte-specific protein 1), CITED4 (CBP/P300 interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 4), Krt19 (keratin 19), FST (follistatin), and MMP3 (matrix metalloproteinase 3) have been shown to be regulated transcriptionally by the activated NF-κB in mammalian cells (Cardozo et al., 2001; Hinz et al., 2002; Hanson et al., 2004; Tian et al., 2005; Hiramitsu et al., 2006; Minami et al., 2006; Huang and Siragy, 2010), suggesting that NF-κB, which is a nuclear transcription factor, may be activated in senescent CEF cells, and in turn, transcriptional expression of downstream genes may be increased. Several secreting proteins, including TNFSF15 [tumor necrosis factor (ligand) superfamily, member 15], MGP (matrix Gla protein), COL4A (collagen type IV), FST, and MMP3 were found in Network #1. Except for COL4A, which has been shown to function in promoting cellular growth (Woodward et al., 2000), TNFSF15, MGP, FST, and MMP3 have been reported to decrease cellular proliferation and to increase cell death (Di Simone et al., 1996; Liu et al., 2000; Newman et al., 2001; Proskuryakov et al., 2003), which are major characteristics shown in senescent CEF cells.

Network #2 contains several secreting factors including MBP (myelin basic protein), CXCL14 (chemokine C-X-C motif ligand 14), IL8 (interleukin 8), BDNF (brain-derived neurotrophic factor), NTS, NPY, and RBP4. The top functions of Network #2 are cell signaling, small molecule biochemistry, and cellular development. Though several signal transduction pathways related to kinases, including MAPK (mitogen-activated protein kinase), ERK (extracellular signal-regulated kinase), PI3K (phosphoinositide 3 kinase), and AMPK (AMP-activated protein kinase) appeared to be associated with the mitogenic responses among molecules in Network #2, the involvement of various signal transduction pathways in this network may be generated due to the inclusion of several secreting proteins. In fact, the arrowheads showing the direction of the interaction were more likely to be pointed toward kinase molecules, meaning that various secreting molecules may stimulate the signaling pathways that are not transcriptionally regulated by kinases. The possible roles of NTS, NPY, and RBP4 in senescent CEF cells are outlined in Supplemental File 3. The CXCL14, in addition to DUSP10 [dual-specificity phosphatase 10; also known as MAP kinase phosphatase 5 (MKP5)], are associated with immune responses and have been shown to suppress cellular proliferation (Zhang et al., 2004; Li et al., 2011). In contrast, MBP, IL8, and BDNF, in addition to PLD (phospholipase D), have been reported to have positive effects on cellular proliferation, which are not compatible to replicative senescence (Almeida et al., 2005; Murphy et al., 2005; Lee et al., 2011; Xu et al., 2011).

Network #3 is closely associated with molecules including tumor necrosis factor α (TNFα), and peroxisome proliferator activated receptor α (PPARA; Figure 3C). The major functions related to Network #3 are organismal development, lipid metabolism, and small molecule biochemistry. Expression of AKR1B10, ALDH1A3, and GSTT1 were increased by the treatment of TNFα in various cell types (Iwata et al., 1999; Li et al., 2002; Banno et al., 2004). The AKR1B10, ALDH1A3, and GSTT1, which were also included in the list of the 10 most upregulated genes in Table 3, are closely related to cellular detoxification processes. Thus, the increased expression of AKR1B10, ALDH1A3, and GSTT1 in senescent CEF cells may be induced by the activation of TNF signaling pathways by the accumulation of metabolic stresses in the aging process. The upregulation of CROT (carnitine O-octanoyltransferase), RBP7, AADAC (arylacetamide deacetylas; esterase), and GSTT1 has been shown to be regulated by activation of PPARA in various cell types (Trickett et al., 2001; Yu et al., 2001; Knight et al., 2008; Sanderson et al., 2010), suggesting a role for PPARA, which was highly activated in senescent CEF cells. The PPARA, which is a nuclear transcription factor and a member of the steroid hormone receptor superfamily, functions in lipid metabolism by regulating gene expression of the protein involved in the cellular free fatty acid uptake and cellular cholesterol trafficking (Akiyama et al., 2002). Gene interactions found in Network #3 showed that senescent CEF cells may have accumulated various metabolic stress and detoxification pathways were highly activated in aged CEF cells.

Comparison of Differential Expression Between Senescent and Immortal DF-1 CEF Cells

Previously, we reported 903 differentially expressed genes mapped by the IPA program in the immortal DF-1 CEF cell line, which exhibits an enhanced growth rate compared with early passage of primary young CEF cells (Kong et al., 2011). Comparing the differ-

Table 5. List of genes regulated oppositely between senescent and immortal DF-1 chicken embryo fibroblast cells

ID	Symbol	Entrez gene name	Senescent[1]	DF-1[2]
AB053322	*LMBR1*	Limb region 1 homolog (mouse)	−1.87	1.19
AB101641	*LSP1*	Lymphocyte-specific protein 1	1.09	−3.02
AF239838	*COL4A1*	Collagen, type IV, α 1	1.53	−5.93
AF355273	*BRCA1*	Breast cancer 1, early onset	−1.23	1.65
AJ249219	*PDLIM3*	PDZ and LIM domain 3	−1.56	1.30
AJ719314	*KIF11*	Kinesin family member 11	−1.16	1.21
AJ719441	*PHKB*	Phosphorylase kinase, β	−1.04	1.26
AJ719646	*NDC80*	NDC80 homolog, kinetochore complex component (yeast)	−1.07	1.29
AJ719849	*MELK*	Maternal embryonic leucine zipper kinase	−1.06	1.10
AJ720093	*BARD1*	*BRCA1*-associated RING domain 1	−2.47	2.09
AJ720394	*RFC3*	Replication factor C (activator 1) 3, 38 kDa	−1.28	2.28
AJ720484	*CKAP2*	Cytoskeleton associated protein 2	−1.53	1.15
AJ720731	*HNRNPK*	Heterogeneous nuclear ribonucleoprotein K	−1.93	2.08
AJ721093	*GSDMA*	Gasdermin A	−1.01	2.12
BX929426	*C16orf88*	Chromosome 16 open reading frame 88	−1.20	1.13
BX930081	*TNFSF15*	Tumor necrosis factor (ligand) superfamily, member 15	1.35	−3.74
BX931642	*NTS*	Neurotensin	1.92	−1.25
BX932394	*SPC25*	SPC25, NDC80 complex component, homolog (yeast)	−1.30	1.00
BX933315	*KHDRBS3*	KH domain, RNA binding, signal transduction associated 3	1.20	−1.42
BX933581	*N4BP2L1*	NEDD4 binding protein 2-like 1	1.37	−1.03
BX935011	*FAH*	Fumarylacetoacetate hydrolase (fumarylacetoacetase)	2.13	−3.89
BX935188	*CCDC109B*	Coiled-coil domain containing 109B	−3.38	1.04
BX950769	*C14ORF79*	Chromosome 14 open reading frame 79	1.32	−1.16
CR353653	*SKA3*	Spindle and kinetochore associated complex subunit 3	−1.04	1.06
CR386255	*LRRC3B*	Leucine rich repeat containing 3B	1.66	−2.88
CR389519	*LRRCC1*	Leucine rich repeat and coiled-coil domain containing 1	−1.12	1.05
CR389919	*TPRN*	Taperin	1.17	−1.72
CR407478	*ARHGEF17*	Rho guanine nucleotide exchange factor (GEF) 17	−1.38	1.85
CR523497	*MAP1A*	Microtubule-associated protein 1A	−2.14	1.17
M87294	*NPY*	Neuropeptide Y	2.61	−3.73
NM_204540	*SOCS2*	Suppressor of cytokine signaling 2	−1.93	1.51
U13676	*GSTT1*	Glutathione S-transferase theta 1	2.18	−2.76
X06749	*MT2A*	Metallothionein 2A	1.66	−1.55
X87609	*FST*	Follistatin	1.64	−4.12
Y13903	*MGP*	Matrix Gla protein	1.18	−3.38

[1]Values are $\log_2$ fold change.

[2]Fold change values were derived from the results of Kong et al. (2011).

entially expressed genes between rapidly growing immortal DF-1 and slowly growing senescent CEF cells may provide better insight into understanding cellular mechanisms responsible for proliferation and lifespan in CEF cells. To attempt to identify significant genes involved in regulating cellular proliferation, the 458 differentially expressed genes in senescent CEF cells were compared with the 903 differentially expressed genes found in immortal DF-1 CEF cells, reported previously (Kong et al., 2011). The results showed that 35 genes overlapped and were oppositely regulated between senescent and immortal DF-1 cells (Tables 5 and 6). Most genes listed in Table 5 are associated with functions of tumorigenesis, cell death, cellular proliferation, and cytoplasm structural organization as shown in Table 6; many genes overlapped in functional categories. The possible functional roles of many of these genes in cellular proliferation and growth for CEF cells were discussed previously (Kong et al., 2011) and elsewhere in this report. The verification of these factors regulating cellular growth, immortalization, and death of CEF cells are currently being investigated.

In summary, global gene expression analysis of slowly growing senescent CEF cells compared with normally growing early passage CEF cells provides insight into the entire genome-wide alterations at the transcriptional level during the cellular aging process of CEF cells. Because downregulated genes predominated in senescent CEF cells and may reflect the reduced cellular biochemical functionalities (major characteristics found in

Table 6. Function of genes regulated oppositely between senescent and immortal DF-1 chicken embryo fibroblast cells

Function	Molecules	No. of molecules
Tumorigenesis	*BARD1*, *BRCA1*, *COL4A1*, *FAH*, *FST*, *GSTT1*, *HNRNPK*, *KHDRBS3*, *KIF11*, *LSP1*, *MELK*, *MGP*, *MT2A*, *N4BP2L1*, *NTS*, *RFC3*, *SOCS2*, *SPC25*	20
Cell death	*BARD1*, *BRCA1*, *CKAP2*, *COL4A1*, *FAH*, *FST*, *GSDMA*, *LSP1*, *MELK*, *MGP*, *MT2A*, *NDC80*, *NPY*, *NTS*, *SPC25*, *TNFSF15*	17
Proliferation of cells	*BARD1*, *BRCA1*, *COL4A1*, *FAH*, *FST*, *HNRNPK*, *KIF11*, *MELK*, *MGP*, *MT2A*, *NPY*, *SOCS2*, *TNFSF15*	14
Organization of cytoplasm	*ARHGEF17*, *KIF11*, *LSP1*, *MAP1A*, *NDC80*, *Pdlim3*, *SPC25*, *TNFSF15*	9

senescent cells), we hypothesize that upregulated genes focused on the functional roles of more active regulators that are involved in inducing senescence. Bioinformatic analyses suggested that genes inducing senescence by increased expression are associated with the suppression of cell cycle and proliferation, progression of cell death including apoptosis, and various secreting factors, and their expression appears to be opposite to the expression of the same genes in immortal CEF cell line, such as DF-1 (Kong et al., 2011). Indeed, the comparison of differentially expressed genes between senescent CEF and immortal DF-1 cells revealed that several genes, such as *BARD1*, *BRCA1*, *FAH*, *FST*, *NPY*, *NTS*, and *TNFSF15*, which regulate cell cycle, proliferation, and death, were oppositely expressed. Further characterization of specific factors to modulate cellular proliferation and regulate the lifespan in CEF cells remains for future studies (e.g., ectopic expression or specific gene knock-down using the small interfering RNA method).

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