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Transcriptome analysis and tumor suppressor requirements of STAT5-induced senescence

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Although it is acknowledged that senescent cells accumulate with age, the molecular mechanisms leading to cell senescence as a function of age remain to be identified. In cell culture models, it has been clearly shown that senescence involves the activation of a DNA damage response secondary to short telomeres or oncogene expression. Oncogenes are altered versions of genes coding for proteins that mediate signals from extracellular factors such as cytokines, growth factors, and hormones. In particular, we show here that constitutive activation of the JAK/STAT5 signaling pathway induces senescence in both mouse and human normal cells. The process involves activation of the p53 and Rb tumor suppressor pathways and mitochondrial dysfunction. Gene expression analysis of STAT5-induced senescence revealed changes in the expression of genes coding for cytokines, proteins in cytokine signaling pathways, and several metabolic enzymes. We discuss a model called senescence-induced senescence, in which cytokines secreted by senescent cells can propagate the process as a function of age.

Keywords: senescence; STAT5; mitochondria; cytokines; p53; Rb; Tel/Jak2

Introduction

Cellular senescence as a DNA damage response

The process of cellular senescence was originally discovered in normal human cells serially passed in culture.[1,2] In this situation, the sequences at the ends of human chromosomes known as telomeres progressively shorten.[3–5] Short telomeres can no longer hide the extremities of the chromosomes, and they thus look like DNA breaks.[6] Senescence is triggered by signals from these short telomeres that use the classic DNA damage response (*DDR*) to activate senescence effector proteins, such as p53 and Rb.[6,7]

Telomere shortening is prevented by the enzyme telomerase, which replenishes telomere sequences during every cell division.[8] Somatic human cells express no or little telomerase, explaining why they have a limited life span in culture.[9] The proliferation of rodent cells is not limited by this mechanism.[10,11] However, cells expressing senescence markers and the DDR do accumulate with age in rodents and primates, although in discrete numbers.[12–15]

If the DDR is the mechanism linking short telomeres and the senescence phenotype, then other causes of DNA damage may also trigger senescence.[7] Oncogenes are also capable of inducing senescence in normal rodent or human cells, and the process does not involve telomere shortening and cannot be rescued by telomerase expression.[13] Oncogenes seem to induce an endogenous pathway of DNA damage that is yet to be characterized.[7,16,17] This pathway may have implications for human aging, because oncogenes are merely activated versions of proteins in signaling pathways that connect extracellular signals to gene expression programs.

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Cytokines and senescence

One interesting characteristic of the gene expression profile of oncogene-induced senescence is the expression of genes coding for cytokines, their receptors, or intracellular signaling proteins.[18–22] Some of these cytokines may be required in an autocrine fashion to regulate the senescence response to oncogenes.[21–23] However, other cytokines may act at a distance, mediating communication between senescent cells and other cells. As a result, cytokines may propagate the senescent phenotype or signal the immune system of a cellular stress condition.[12,18,24–27] Cytokines secreted by senescent cells may also trigger an innate immune response aimed at the elimination of senescent cells.[25]

The close link between cytokines and senescence suggests that cytokine receptors and the signaling proteins they activate may play a role in senescence as well. Cytokines activate many different signaling pathways, and the picture emerging from studies of oncogene-induced senescence is that constitutive activation of cytokine and growth factor signaling pathways also induces senescence. This is true for the Ras/ERK signaling pathway,[28–40] the phosphatidylinositol 3-kinase/AKT[41–43] pathway, the p38 MAPK pathway,[33,44–50] and the Jak/STAT5 pathway, which was recently linked to cellular senescence in our laboratory.[7,51,52]

Materials and methods

Cell culture, transfection, and retroviral infection

Normal human diploid fibroblast IMR90 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin G–streptomycin sulfate (GIBCO). Primary mouse embryo fibroblasts (*MEFs*) were obtained from 13.5-day wild-type, p53 null, or ARF null embryos as described elsewhere.[37] Retroviral plasmids pBabe, pBabeRasV12, and pBabeSTAT5A1∗6 are described in Refs. 51 and 52. pBabeTel/Jak2(4-17) was obtained by subcloning the blunted NheI-EcoRI fragment from pGLETV6Jak2(4-17)[53] (kindly provided by P. Marynen) into pBabe digested with SnaBI. Retrovirus-mediated gene transfer, cell proliferation analysis (growth curves and [^{3}H]thymidine incorporation assays), and senescence-associated β-galactosidase assays (*SA–β-Gal*) were performed as described previously.[51,52]

Western blotting and fluorescence microscopy

Briefly, 20 μg of cell lysate prepared in sodium dodecyl sulfate (*SDS*) sample buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol) was separated by SDS-polyacrylamide gel electrophoresis and then transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The primary antibodies used were the following: anti-p19^{ARF} (1:500; Novus, Littleton, CO, USA), anti-phospho-p53^{S15} (9286; 1:2000; Cell Signaling, Danvers, MA, USA), anti-p21 (C-19; 1:750; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Rb (G3-245; 1:250; BD Pharmingen, Franklin Lakes, NJ), anti-cyclin A (C-19; 1:500; Santa Cruz Biotechnology), anti-phospho-STAT5^{Y694} (9351; 1:1000; Cell Signaling), anti-tubulin (B-512; 1:5000; Sigma, St. Louis, MO, USA), and anti-promyelocytic leukemia antigen (anti-PML; PG-M3; 1:200; Santa Cruz Biotechnology). Signals were revealed after incubation with secondary antibodies coupled to horseradish peroxidase and by using enhanced chemiluminescence (Amersham, Buckinghamshire, UK). For indirect immunofluorescence and microscopy, MEFs infected with the control empty vector pBabe, pBabeRasV12, or pBabeSTAT5A1∗6 were plated on coverslips in six-well plates. Cells were fixed with 4% paraformaldehyde and permeabilized by incubating for 5 min in phosphate-buffered saline (*PBS*)–3% bovine serum albumin (*BSA*) plus 0.2% Triton X-100. After three washes with PBS–3% BSA, cells were incubated for 1 h with anti-PML (PG-M3; 1:200; Santa Cruz Biotechnology), washed again three times with PBS–3% BSA, incubated for another hour with Alexa 568 secondary antibody (Molecular Probes, Eugene, OR, USA), washed three times with PBS, and then incubated for 10 min in 300 nM 4′,6-diamidino-2-phenylindole. Images were obtained using a Nikon Eclipse TE-200U microscope and the MetaMorph software (Universal Imaging Corp., Sunnyvale, CA, USA).

Mitochondrial membrane potential and reactive oxygen species staining

Mitochondrial mass was estimated by staining cells with MitoFluor Green (M-7502; Molecular Probes). Cells were incubated for 15 min with 100 nM

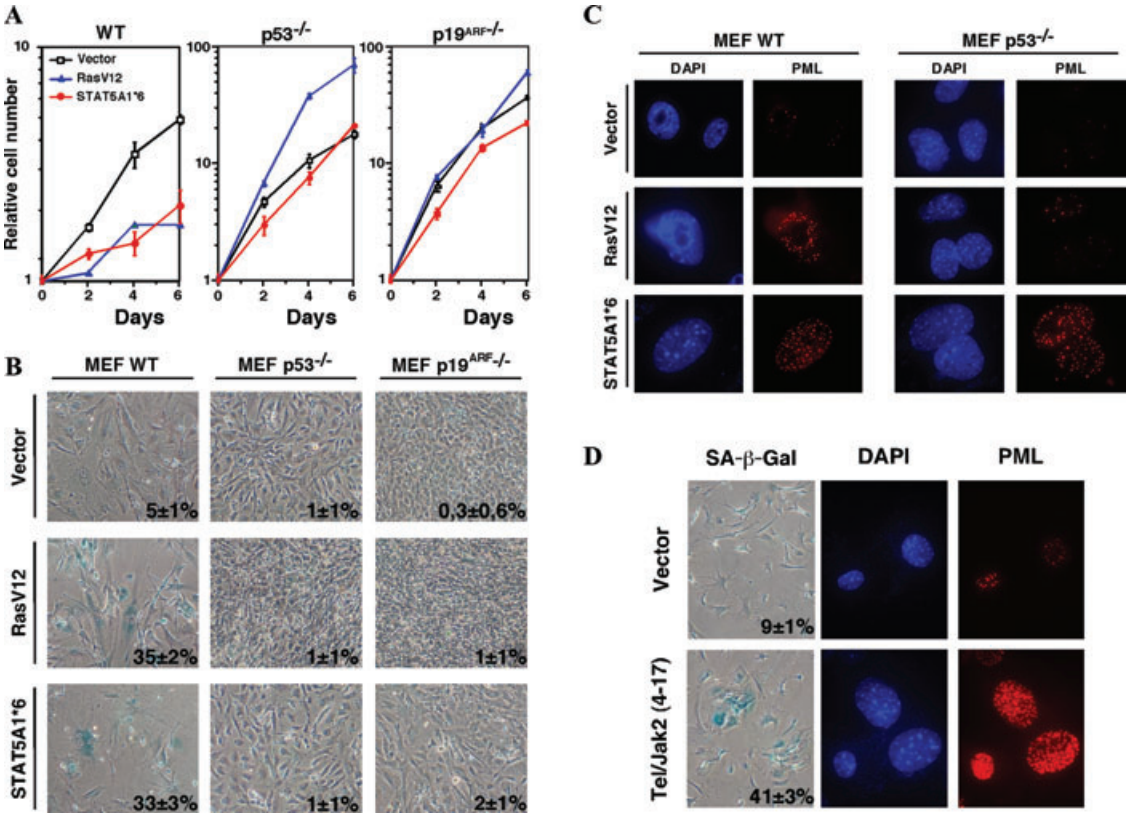

Figure 1. STAT5A activation induces senescence in mouse cells. (**A**) Growth curves of wild-type (*WT*), $p53^{-/-}$, and $p19^{ARF-/-}$ MEFs infected with control vector, RasV12, or STAT5A1*6. (**B**) SA–β-Gal of WT, $p53^{-/-}$, and $p19^{ARF-/-}$ MEFs infected with empty vector, RasV12, or STAT5A1*6. (**C**) PML immunofluorescence in WT and $p53^{-/-}$ MEFs with control vector, RasV12, or STAT5A1*6. (**D**) SA–β-Gal staining and PML immunofluorescence of WT MEFs infected with empty vector or Tel/Jak2(4-17). In panels **B** and **D**, the percentages and standard deviations of SA–β-Gal-positive cells are indicated at the bottom right of each SA–β-Gal image. Data represent three independent experiments performed with cells collected 6 days postselection. *DAPI*, 4′,6-diamidino-2-phenylindole.

MitoFluor Green and washed with Hank's balanced salt solution (GIBCO). To measure superoxide, cells were treated with 1 μM dihydroethidium (Molecular Probes). Cell nuclei were stained with 4 μg/ml Hoechst stain (Molecular Probes). The intensity of staining was analyzed by fluorescence-activated cell sorting.

RNA preparation and microarray

Total RNA from IMR90 cells infected with control empty vector (pBabe) or pBabe-STAT5A1∗6 was isolated using UltraSpec (Biotecx Labs, Houston, TX, USA) at day 8 postselection. Purified RNA was then analyzed using the Human Genome U133 microarray set (Affymetrix, Santa Clara, CA, USA) to compare mRNA levels (Genome Quebec, Montreal, Quebec, Canada).

Real-time PCR was performed as described elsewhere,[52] and the sequences of the primers are available upon request.

Results

Induction of p53-dependent senescence in MEFs by constitutive STAT5 signaling

We showed before that the constitutively active alleles of STAT5A and -B (STAT5A1∗6 and STAT5B1∗6) were sufficient to trigger a senescence program in human diploid fibroblast strains IMR-90 and BJ.[51,52] We show here that primary MEFs also undergo cell cycle arrest (Fig. 1A), with markers of cellular

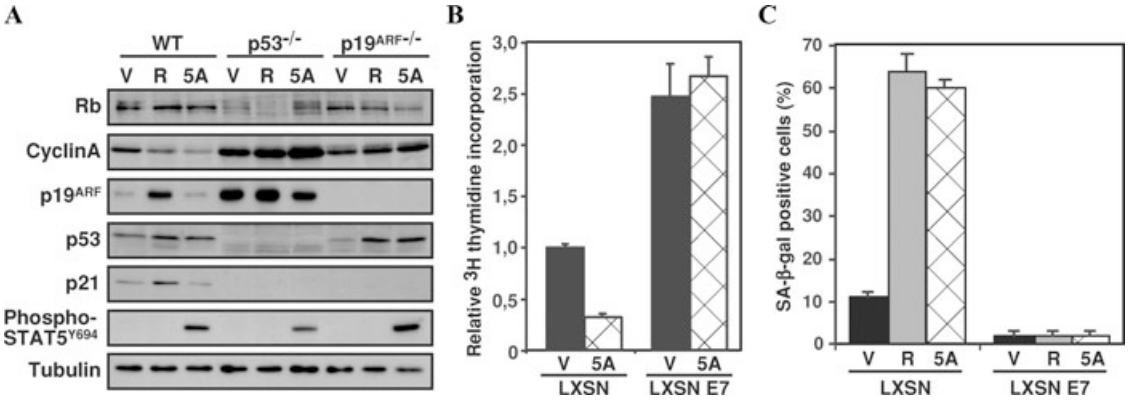

Figure 2. Cell cycle control of STAT5-induced senescence in MEFs. (**A**) Western blot analysis of cell cycle regulators and phospho-STAT5^{Y694} in wild-type (*WT*), p53$^{-/-}$, and ARF$^{-/-}$ MEFs infected with empty vector (V), RasV12 (R), or STAT5A1*6 (5A). (**B**) [^{3}H]thymidine incorporation of LXSN or LXSN E7 MEFs infected with either empty vector or STAT5A1*6. (**C**) SA–β-Gal staining of LXSN- or LXSNE7-infected MEFs containing the empty vector, RasV12, or STAT5A1*6. Data represent three independent experiments performed with cells collected 6 days postselection.

senescence (Fig. 1B and C) when forced to express a constitutively active allele of STAT5A (*caS5A*) or RasV12 by retroviral gene transfer. The process required p53 or the p53 activator p19^{ARF}, because it was totally prevented in MEFs from p53 or p19^{ARF} null mice (Fig. 1A–C). To investigate whether we could also trigger senescence by activating JAK/STAT5 signaling upstream of STAT5, we used a Tel/Jak2 fusion protein.[54] Similar to caS5A, Tel/Jak2(4-17) was also sufficient to trigger senescence cell cycle arrest in MEFs (Fig. 1D).

Next, we characterized the status of the p53 and the Rb pathways in MEFs expressing RasV12, caSTAT5A, or a control vector. Expression of RasV12 or caS5A reduced Rb phosphorylation, as seen by faster migration of Rb in the SDS–polyacrylamide gel electrophoresis assay. Also, these oncogenes reduced the levels of E2F target genes (*i.e.*, cyclin A) in wild-type MEFs (Fig. 2A) but not in MEFs with a disabled p53 pathway. Both RasV12 and caS5A stabilized p53, but the levels of the p53 target p21 were higher in cells expressing RasV12. Intriguingly, caS5A did not induce p19^{ARF}, although p19^{ARF} was required for the senescence response. This suggests that basal levels of p19^{ARF} perform an essential function in the senescence pathway. Altogether, these results confirm the essential requirement of the p53 pathway for oncogene-induced senescence in MEFs, but they reveal interesting biochemical differences between caS5A and RasV12.

To address the Rb pathway requirement in MEFs, we coinfected caS5A- or RasV12-expressing cells with the viral oncoprotein E7, which inactivates all three members of the Rb family.[55] E7 blocked the ability of either caS5A or RasV12 to induce a senescence cell cycle arrest (Fig. 2B and C). Together, the results support an essential role for both the p53 and Rb tumor suppressor pathways in STAT5A-induced senescence in MEFs.

Mechanisms of cS5A-induced senescence in human cells

For human fibroblasts, we have reported that caS5A induces senescence via both p53 and Rb.[51,52] Mechanistically, p53 is induced via a DDR that involves ATM, ATR, Chk1, and Chk2.[51] The Rb pathway can be activated by an independent pathway that involves the downregulation of Myc.[52] In searching for mechanisms to explain how caS5A may damage the DNA, we found that cells expressing caS5A produce a large amount of hydrogen peroxide.[7] caS5A-expressing cells were also labeled very strongly with a superoxide-sensitive probe, dihydroethidium (Fig. 3A). In addition, these cells had an increase in mitochondrial mass as measured with the probe MitoFluor Green (Fig. 3B). These results suggest that caS5A causes a mitochondrial dysfunction leading to reactive oxygen species production, oxidative DNA damage, and activation of the DDR.

Table 1. Genes coding for cytokines, growth factors, signaling proteins, and metabolism are upregulated in cellular senescence[a]

Cellular function	Gene abbreviation	Gene name	STAT5A1*6 (fold induction vs control)[a]	RasV12[b]
Cytokines and growth factors	THBS1	Thrombospondin 1	+5, 0	
	THBS2	Thrombospondin 2	+2, 8	
	IL-8	Interleukin-8	+3, 0	+ (19, 21, 22)
	IGFBP3	Insulin-like growth factor binding protein 3	+1, 8	
	IGFBP5	Insulin-like growth factor binding protein 5	+3, 4	
	NOV	Nephroblastoma overexpressed gene (IGFBP9)	+2, 9	
	VEGFC	Vascular endothelial growth Factor C	+2, 8	+ (69)
	SGNE1	Secretory granule neuroendocrine protein 1 (7B2 protein)	+2, 9	
	INHBA	Inhibin A	+2,1	
	GMFB	Glial maturation factor beta	+2, 9	
	TIMP3	Tissue inhibitor of metalloproteinase 3	+2, 1	
	Serpines	Serpin B2	+3, 4	
		Serpine E1 (PAI-1)	+2, 0	+ (20, 56)
		Serpine E2	+3, 3	
	TNFRSF11B	Tumor necrosis factor receptor superfamily 11B/osteoprotegerin	+6, 8	
	IL-1R1	IL-1 receptor type 1	+2, 6	
	IL-7R	IL-7 receptor	+3, 1	
	CXCL1	Chemokine ligand 1/GRO-a	+2, 4	
	CXCL12	Chemokine ligand 12	+2, 1	
Signal transduction	SOCS1	Suppressor of cytokine signaling 1	+7, 9	
	SOCS2	Suppressor of cytokine signaling 2	+3, 0	
	SOCS4	Suppressor of cytokine signaling 4	+2, 8	+*
	SOCS5	Suppressor of cytokine signaling 5	+1, 9	
	CISH	Cytokine-inducible SH2-containing protein	+3, 1	
	PEA15	Phosphoprotein-enriched in astrocytes	+2, 2	+ (29)
	GSK3B	Glycogen synthase kinase 3B	+2, 2	
Chromatin architecture	HMGA2	High-mobility group AT hook 2	+2, 6	+ (70)
Metabolism	HK1	Hexokinase 1	+2, 1	
	UGDH	UDP-glucose dehydrogenase	+2, 8	+ (71)
	GFPT1	Glutamine–fructose-6-phosphate transaminase 1	+2, 3	
	GFPT2	Glutamine–fructose-6-phosphate transaminase 2	+2, 0	
	UAP1	UDP *N*-acetyl glucosamine phosphorylase	+2, 4	
	NDUFA5	NADH dehydrogenase 1α subcomplex 5	+2, 3	
	NDUFS7	NADH dehydrogenase Fe-S protein 7		+*
	PCBD	Pyruvoyl-tetrahydropterin synthase	+3, 1	
	PAPSS2	Phosphoadenosine 5′ phosphosulfate transferase	+2, 3	
	GLRX	Glutaredoxin	+3, 2	+*
	EKI1	Ethanolamine kinase	+2, 4	

Continued.

Table 1. *Continued*

Cellular function	Gene abbreviation	Gene name	STAT5A1*6 (fold induction vs control)[a]	RasV12[b]
	MTRR	Methyl tetrahydrofolate-homocysteine methyltransferase reductase	+4, 1	
	MGST2	Microsomal glutathione *S*-transferase	+2, 4	
	SOAT1	Sterol *O*-acyltransferase	+2, 9	
	MVK	Mevalonate kinase	+3, 4	
	ENO2	Enolase 2	+2, 4	
	ME1	Malic enzyme 1	+2, 2	
	UGCG	UDP glucose–ceramide glucosyl transferase	+2, 9	
	GLDC	Glycine dehydrogenase	+7, 2	
	CH25H	Cholesterol 25 hydroxylase	+9, 4	+*
	ALDH1A3	Aldehyde dehydrogenase 1 family member 3	+12, 7	

[a]The Human Genome U133 microarray set (Affymetrix) was used to compare mRNA expression levels in pBabe- and pBabeSTAT5A1*6-infected IMR90 cells at day 8 postselection.
[b]The reference(s) in which a similar gene expression change in cell senescence was reported is indicated for each gene, when available (see Ref. 20). *, the array of RasV12-induced senescence is the same as that reported in Ref. 20; however, the data reported here were not published previously.

Gene expression changes in cells expressing caS5A

The mechanism by which STAT5 and other signaling proteins actually trigger DNA damage, mitochondrial dysfunction, and activation of Rb remains to be characterized. However, the gene expression changes induced by the aberrant activation of these proteins may shed light on the process. We have characterized the gene expression profile of STAT5-induced senescence. In Table 1 we highlight the most notable gene expression changes in caS5A-induced senescence. Careful inspection of the data indicates that two categories of genes are enriched in senescent cells. First, many upregulated genes code for cytokines and proteins in cytokine signaling pathways. This is consistent with reports indicating that the cytokines interleukin-6 (*IL-6*), IL-8, and insulin-like growth factor (*IGF*) BP7 are required for senescence induction in response to oncogenes or short telomeres.[21–23] Among the upregulated

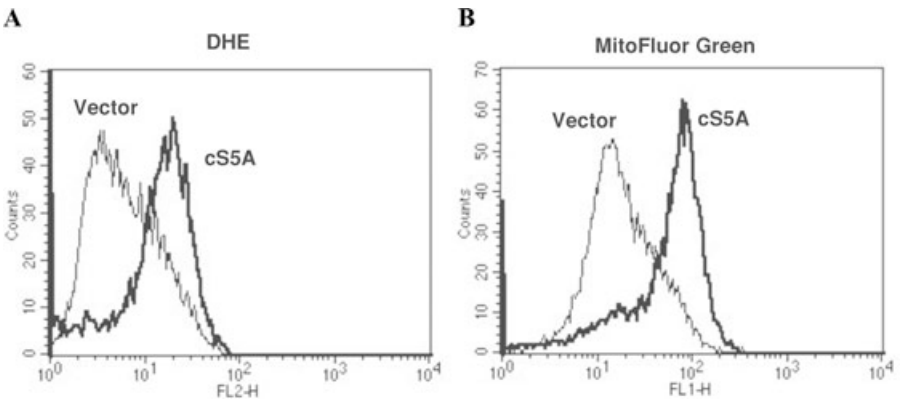

Figure 3. Mitochondrial changes in caS5A-expressing cells. Graphs show the fluorescence intensities of cells analyzed by flow cytometry and stained with dihydroethidium (*DHE*) (A) or MitoFluor Green (B).

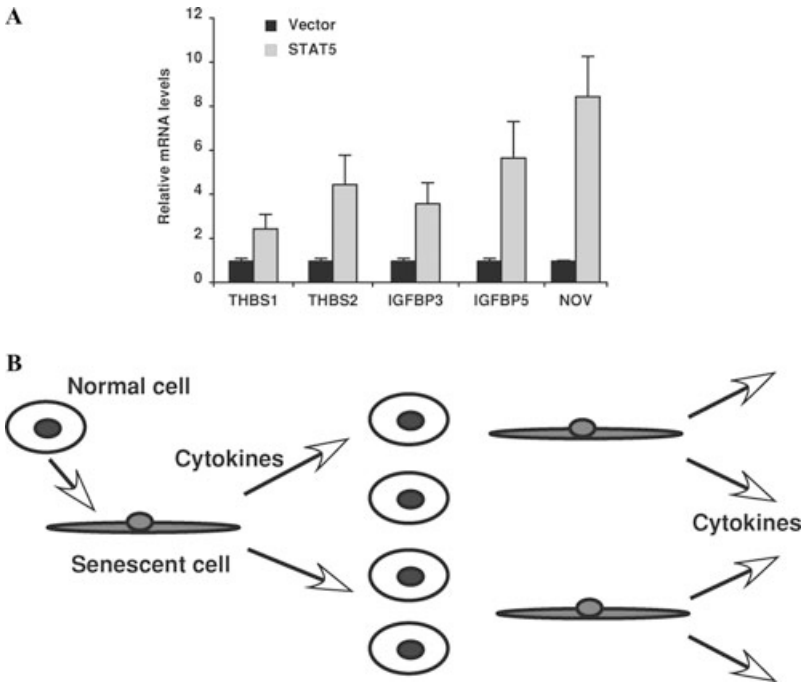

Figure 4. **Cytokines and senescence. (A) Real-time RT-PCR of cytokines upregulated in caS5A-induced senescence (see Table 1). RNA from IMR-90 cells infected with a control empty vector or caS5A was used for quantitative RT-PCR. *THBS1*, thrombospondin 1; *THBS2*, thrombospondin 2; *IGFBP3*, IGF-binding protein 3; *IGFBP5*, IGF-binding protein 5; *NOV*, nephroblastoma overexpressed gene. (B) Senescence-induced senescence. In this model cytokines secreted by senescent cells can propagate the process, resulting in proliferation arrest in dividing tissues or cell malfunction in postmitotic cells.**

genes, serpine E1/PAI-1, a long-used marker of senescence, is a known p53 target gene responsible for activation of GSK3β and degradation of cyclin D1.[56] Interestingly, STAT5 triggered the upregulation of IL-8, CXCL1/GRO-a, and osteoprotegerin genes. These genes have been previously identified as part of the senescence-associated secretory phenotype of replicative senescence and RasV12-induced senescence.[19] Furthermore, IL-8 is in part responsible for a paracrine mechanism promoting invasiveness of cultured premalignant epithelial cells.[19] We confirmed the expression of some of these cytokines by real-time reverse transcription-PCR (*RT-PCR*), thus validating the array data (Fig. 4A). These altered expression levels of cytokines and growth factors during STAT5-induced senescence may explain the development of age-related cancer through disturbances in the tissue microenvironment. Second, the expression of many metabolic enzymes is upregulated in caS5A-induced senescence, suggesting a metabolic reprogramming of these cells.

Discussion

The accumulation of senescent cells in old organisms raises important questions about the origins and the effects these cells may have in tissue physiology. Although the senescence program acts as an efficient tumor suppressor mechanism *in vivo*, senescent cells may have detrimental effects for an organism in the long term.[12,13,57] The relationship between cytokine signaling and senescence is intriguing and so far has been explained in the context of tumor suppression.[21–23,58] However, the discovery that aberrant JAK/STAT5 signaling induces senescence[51,52] could be relevant to aging in light of the ample evidence linking growth hormone and aging.[59–61] Growth hormone, as well as other cytokines, signals via STAT5,[62–65] suggesting that modulation of STAT5 signaling could induce the same prolongevity effects seen in mice with a disabled somatotropic axis. The role of STAT5 in aging has been poorly studied, but it has been reported

that aberrant STAT5 signaling correlates with immunosenescence.[66] Intriguingly, fibroblast growth factor 2, a hormone induced by starvation,[67] inhibits growth hormone signaling by reducing the levels of STAT5.[68] A similar mechanism may play a role in the antiaging effects of caloric restriction.

None of the signaling proteins linked to senescence so far is a unique senescence regulator. All of them play a role in normal signaling pathways that regulate cell growth, cell differentiation, and the normal response to cytokines and growth factors. Their senescence-inducing abilities are activated when their activity bypasses a certain threshold. This may happen accidentally with oncogenic mutations, but such mutations are such a rare event that this cannot explain the accumulation of a sufficient number of senescent cells with aging. The discovery that several cytokines are sufficient to trigger senescence[18,19,21–23,27] suggests a model we call senescence-induced senescence. In this model, senescent cells may arise randomly by mutations or telomere damage. However, these senescent cells, if they persist, can recruit other cells into senescence by secreting prosenescence cytokines (Fig. 4B). This will initiate a vicious cycle that could explain the accumulation of senescent cells with age. This model suggests that mechanisms that modulate cytokine signaling or control the clearance of senescent cells inhibit aging and may differ between species with different life spans.

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Conflicts of interest

The authors declare no conflicts of interest.

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