

ORIGINAL ARTICLE

Cytokine expression and signaling in drug-induced cellular senescence

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Cellular senescence guards against cancer and modulates aging; however, the underlying mechanisms remain poorly understood. Here, we show that genotoxic drugs capable of inducing premature senescence in normal and cancer cells, such as 5-bromo-2'-deoxyuridine (BrdU), distamycin A (DMA), aphidicolin and hydroxyurea, persistently activate Janus kinase–signal transducer and activator of transcription (JAK/STAT) signaling and expression of interferon-stimulated genes (ISGs), such as *MXI*, *OAS*, *ISG15*, *STAT1*, *PML*, *IRF1* and *IRF7*, in several human cancer cell lines. JAK1/STAT-activating ligands, interleukin 10 (IL10), IL20, IL24, interferon γ (IFN γ), IFN β and IL6, were also expressed by senescent cells, supporting autocrine/paracrine activation of JAK1/STAT. Furthermore, cytokine genes, including proinflammatory IL1, tumor necrosis factor and transforming growth factor families, were highly expressed. The strongest inducer of JAK/STAT signaling, cytokine production and senescence was BrdU combined with DMA. RNA interference-mediated knockdown of JAK1 abolished expression of ISGs, but not DNA damage signaling or senescence. Thus, although DNA damage signaling, p53 and RB activation, and the cytokine/chemokine secretory phenotype are apparently shared by all types of senescence, our data reveal so far unprecedented activation of the IFN β –STAT1–ISGs axis, and indicate a less prominent causative role of IL6–JAK/STAT signaling in genotoxic drug-induced

senescence compared with reports on oncogene-induced or replicative senescence. These results highlight shared and unique features of drug-induced cellular senescence, and implicate induction of cancer secretory phenotype in chemotherapy.

Oncogene (2010) 29, 273–284; doi:10.1038/onc.2009.318; published online 5 October 2009

Keywords: cytokines; JAK/STAT signaling; interleukins; cellular senescence; 5-bromo-2'-deoxyuridine; distamycin A

Introduction

Human cells proliferating *in vitro* randomly withdraw from the cell cycle and enter a state termed replicative cellular senescence (Hayflick and Moorhead, 1961). This complex phenotype is characterized by persistent cell cycle arrest, morphological and functional features (Campisi, 2005), including profound changes in cell secretory phenotype (Kuilman and Peeper, 2009). Replicative senescence is primarily caused by telomeric DNA attrition, which can be accelerated, for example, by oxidative stress (von Zglinicki, 2002). The progression towards replicative senescence is accompanied by gradual increase of the tumor suppressor p53, cyclin-dependent kinase inhibitors, p21^{WAF1/CIP1} (p21) and p16^{INK4a} (p16), and decline of growth-promoting factors such as c-Fos (Bringold and Serrano, 2000). The absence or abrogation of senescence is frequently observed under conditions compromising the function of p53 and RB tumor suppressor pathways, consistent with their key roles in cell cycle arrest and development of the senescent phenotype (Campisi, 2005; Mallette and Ferbeyre, 2007).

Of late, several forms of premature senescence independent of the proliferative history and telomere shortening have been described (Schmitt, 2007). Oncogene-induced senescence can be elicited by exposure of cells to aberrant mitogenic or oncogenic signals, such as mutational activation or overexpression of Ras, mos, cdc6 (cell division cycle 6), cyclin E, STAT5 (signal transducer and activator of transcription 5), etc.

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Received 8 September 2008; revised 9 August 2009; accepted 2 September 2009; published online 5 October 2009

(Serrano *et al.*, 1997; Bartkova *et al.*, 2006; Di Micco *et al.*, 2006; Collado *et al.*, 2007; Mallette *et al.*, 2007). Chronic subtoxic doses of stress-inducing compounds such as ethanol or H₂O₂ can cause stress-induced senescence (Toussaint *et al.*, 2002). Drug-induced senescence can be promoted by a variety of chemically and functionally unrelated DNA-damaging anticancer agents, such as doxorubicin (Chang *et al.*, 1999), camptothecin (Han *et al.*, 2002), 5-aza-2'-deoxycytidine (Timmermann *et al.*, 1998; Kulaeva *et al.*, 2003), aphidicolin (APH) and hydroxyurea (HU) (Yogev *et al.*, 2006), or halogenated nucleotide analogs such as 5-bromo-2'-deoxyuridine (BrdU) (Michishita *et al.*, 1999; Minagawa *et al.*, 2005). Despite the fact that oncogenic or stress stimuli do not promote telomere shortening, prematurely senescent cells share similar characteristics with cells undergoing replicative senescence. Overall, cellular senescence represents a universal growth arrest program, which can be triggered by diverse stimuli.

Importantly, markers characteristic of cells undergoing senescence *in vitro* were also found in preinvasive lesions of multiple types of human tumors (Michaloglou *et al.*, 2005; Bartkova *et al.*, 2006; Di Micco *et al.*, 2006; Collado *et al.*, 2007; Acosta *et al.*, 2008; Kuilman *et al.*, 2008), supporting a view that cellular senescence acts as a tumorigenesis barrier (Sager, 1991; Halazonetis *et al.*, 2008). On the other hand, senescent cells in tissues have also been suggested to facilitate tumor growth (Krtolica *et al.*, 2001; Campisi, 2005; Parrinello *et al.*, 2005) through secreted factors that can promote tumorigenesis. For example, the ability of some cytokines to induce DNA damage during their chronic administration *in vitro* (Moiseeva *et al.*, 2006) indicates their potential contribution to genome destabilization due to local effects of secreted compounds. Such vicious circle would also explain the coincidence of chronic inflammation (to which the secretion of proinflammatory cytokines by senescent cells can contribute) with predisposition to malignancy (Coussens and Werb, 2002; Lin and Karin, 2007). To elucidate these issues, new insights into cytokine secretion under various senescence-promoting conditions and its influence on pathophysiology of senescent cells are needed. To contribute to such efforts, we have examined the effects of several genotoxic, senescence-inducing compounds, such as halogenated deoxyuridines, thymidine, camptothecin, distamycin A (DMA), HU and APH, on cytokine/chemokine signaling and its potential role in premature cellular senescence.

Results

BrdU and DMA synergistically activate and induce STAT1

Long-term administration of BrdU causes premature cellular senescence in various cell lines (Michishita *et al.*, 1999; Minagawa *et al.*, 2005), synergistically with DMA, an AT-binding ligand (Suzuki *et al.*, 2002). Similar to other types of senescence, BrdU + DMA-induced senes-

cence (BDIS) is also accompanied by elevation of PML nuclear bodies (Janderova-Rossmeslova *et al.*, 2007). While searching for a stimulus capable of activating the interferon-regulated PML gene during premature senescence (Chelbi-Alix *et al.*, 1995), we found elevated levels of activated forms of STAT1 in cells undergoing BDIS. STAT1 phosphorylated on tyrosine 701 and serine 727 gradually increased during long-term treatment of HeLa cells with the senescence-inducing mixture of BrdU/DMA (each 10 μ M; Figure 1a). The presence of both STAT1 forms was clearly observable at day 4, peaking between days 6 and 8 and then slowly decaying, accompanied by elevation of total STAT1 (Figure 1a). In contrast, the increase of total and Tyr701/Ser727-phosphorylated STAT1 was almost undetectable when either drug was used separately (Figure 1b; only day 6 of the treatment is shown). Even 10-fold higher concentration of BrdU (100 μ M) did not outreach the effect of combined BrdU + DMA (Figure 1b), indicating synergistic action of both drugs on STAT1 expression and phosphorylation. Similar to HeLa cells, synergistic induction and activation of STAT1 with BrdU and DMA was found in A549 (Figure 1c) and U2OS cells (Figure 1d). However, in H1299 cells, DMA alone, but not BrdU even at high concentrations, induced total STAT1 and Tyr701/Ser727 phosphorylation (Supplementary Figure 1a), whereas MDA-MB-468 and HS913T cells were less sensitive to both drugs at concentrations effective in other cell lines (Supplementary Figures 1b and c).

5-Bromo-2'-deoxyuridine + DMA-treated HeLa cells displayed the activation of STAT1 concomitantly with proliferation arrest (Supplementary Figure 2g), enhanced senescence-associated β -galactosidase positivity (Supplementary Figures 1d and e), and induction and activation of tumor suppressors and cell cycle inhibitors (increased levels of p53, p21, phosphorylation of serine 15 of p53 and hypophosphorylation of RB; Supplementary Figure 2a). However, U2OS cells treated with 100 μ M BrdU developed senescence-like phenotype with all markers present (Supplementary Figures 1f, g, 2c and i), yet without simultaneous activation of STAT1, indicating that STAT1 activation is not a universal feature of cellular senescence (Figure 1d).

Next, we examined how general is STAT1 activation in response to diverse genotoxic compounds. When halogenated nucleoside derivative 5-chloro-2'-deoxyuridine was used instead of BrdU, it synergized with DMA and activated STAT1 similar to BrdU (Supplementary Figure 1h). Other genotoxic compounds known to induce DNA replication stress and senescence in cancer cells, such as APH, HU (Yogev *et al.*, 2006), Supplementary Figures 1i–l), thymidine and camptothecin (Han *et al.*, 2002; Engstrom and Kmiec, 2007), data not shown) also activated and induced STAT1. APH or HU used individually at concentrations inducing an obvious senescent phenotype in HeLa cells induced phosphorylation of STAT1 at both sites and induction of total STAT1 to levels (Figure 1e) higher than that induced by BrdU or DMA alone, but not to the level induced by combined BrdU + DMA. Unexpectedly,

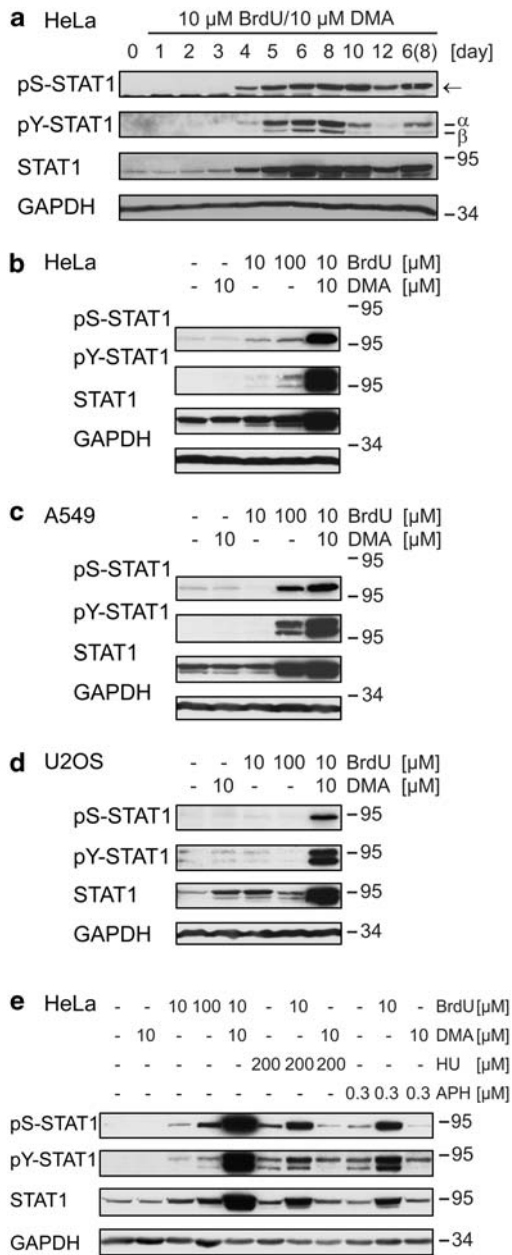


Figure 1 Genotoxic drugs induce expression and phosphorylation of signal transducer and activator of transcription 1 (STAT1) in various cell lines. **(a)** Immunoblotting analysis of a time-course of STAT1 expression and its phosphorylation on serine 727 (pS, specific band is marked by arrow) and tyrosine 701 (pY; α , β : STAT1 α and β isoforms detected) in HeLa cells treated by 10 μ M 5-bromo-2'-deoxyuridine (BrdU) and 10 μ M distamycin A (DMA). Note that increased expression and phosphorylation of STAT1 remained stable for next 2 days of cultivating in the absence of BrdU + DMA in culture medium (last lane). Immunoblot detection of total and phosphorylated forms of STAT1 in HeLa **(b)**, A549 **(c)** and U2OS **(d)** cells after 6 days of treatment. **(e)** Immunoblot comparison of the effect of BrdU, DMA, hydroxyurea, aphidicolin and their combinations on STAT1 expression and phosphorylation. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control.

other AT-binding agents, such as the distamycin-related netropsin or the DNA intercalator Hoechst33258, showed no or weak effects on STAT1 (Supplementary

Figure 1h). APH (0.3 μ M) and HU (200 μ M) combined with 10 μ M BrdU or 10 μ M DMA had additive, rather than synergistic effects on STAT1 phosphorylation (Figure 1e). Thus, STAT1 is activated by diverse chemical inducers of senescence in a cell line-dependent manner. To gain further insights into the biology of this phenomenon, we selected the synergistic combination of BrdU + DMA.

Cytokine expression in HeLa cells exposed to BrdU + DMA

As activation of STAT1 can be achieved by paracrine/autocrine signaling of diverse cytokines through membrane receptors, we next considered such scenario. The presence of secreted cytokines was supported by the ability of the medium conditioned by senescent HeLa cells to induce interferon-stimulated proteins, PML and STAT1, in untreated HeLa cells (Figure 2a). Moreover, using the RT² Profiler PCR Array System (SuperArray Bioscience Corp., Frederic, MD, USA) ('Interferons and receptors' and 'Common cytokines'; see Supplementary Materials and methods), we found increased transcript levels of several cytokine species, including a transforming growth factor family member inhibin β A (~1400-fold), interleukin 8 (IL8) (~400-fold), IL24 (~300-fold), IL6 (~150-fold), a TNF (tumor necrosis factor) family member CD70 (TNFSF7; ~130-fold), transforming growth factor- α (~100-fold), IL1 β (~40-fold), interferon β (IFN β) (~15-fold) and IFN γ (more than threefold) (Tables 1 and 2; Supplementary Tables 1 and 2). We conclude that mRNA levels of several cytokines, including ligands capable of receptor-mediated activation of STAT1 (IFN β , IFN γ , IL6, IL20, IL24 (Commins *et al.*, 2008)), were indeed elevated in BDIS.

STAT1-activating ligands IFN β and IL6 are secreted by senescent HeLa cells

The expression of IFN β , the major STAT1-activating ligand, was confirmed in separate time-course experiments. Whereas IFN β mRNA was induced 2.6-fold and 47-fold in BrdU- and DMA-treated HeLa cells, respectively, the BrdU + DMA combination caused a gradual increase of IFN β mRNA to 268-fold elevation by day 6, as assessed by quantitative reverse transcription PCR (Figure 2b), consistent with synergistic effects of the combined treatment. The IFN β mRNA in cells treated with high BrdU (100 μ M) alone increased less, yet significantly (16-fold by day 6; Figure 2b). The BrdU + DMA-induced elevation of IFN β mRNA was also reproduced in A549 cells (Figure 2c). Finally, enhanced IFN β protein in culture medium of BrdU + DMA-treated HeLa cells was detected by ELISA (enzyme-linked immunosorbent assay) (Figure 2d) upon a 6-day culture, compared with parallel controls without BrdU/DMA.

Interleukin 6, though not the dominant inducer of STAT1, can contribute to STAT1 activation (Qing and Stark, 2004). We assessed secretion of the IL6 protein after treatment of HeLa cells with DMA or BrdU + DMA by immunoblotting (Figure 3a) and cytometric

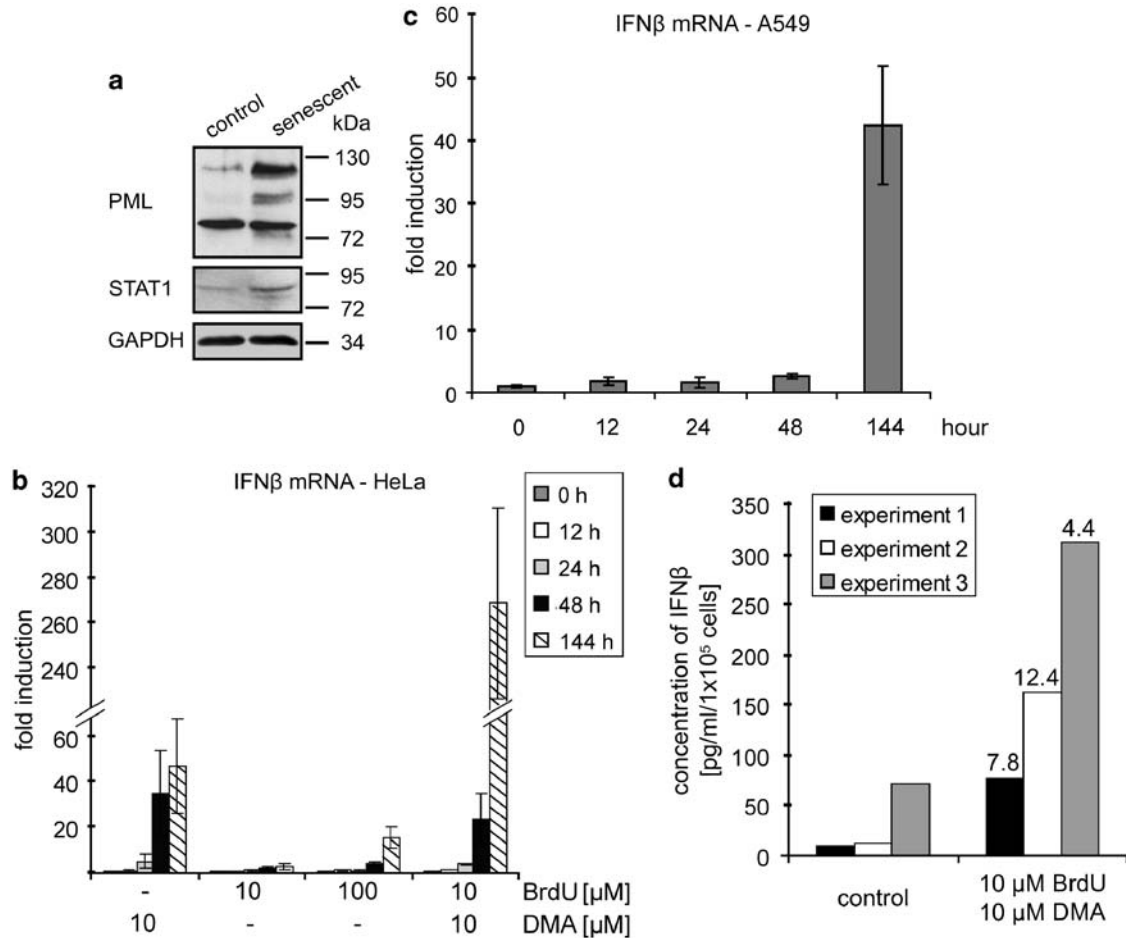


Figure 2 5-Bromo-2'-deoxyuridine plus distamycin A (BrdU + DMA) treatment induces expression of interferon- β (IFN β). (a) Immunoblot detection of PML and signal transducer and activator of transcription 1 (STAT1) in control HeLa cells grown for 4 days in normal medium (control) and medium conditioned by BrdU + DMA-treated, senescent HeLa cells (senescent; for details see Supplementary Materials and methods). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) serves as loading control. IFN β mRNA levels quantified by quantitative reverse transcription PCR in HeLa cells (b) treated by BrdU and DMA and in A549 cells (c) treated by the combination of 10 μ M BrdU and 10 μ M DMA for specified time periods. The values representing average from two (b) and three (c) independent experiments are shown as a fold induction relative to control; error bars represent standard error. (d) Enzyme-linked immunosorbent assay (ELISA) of IFN β secreted into medium by control and BrdU + DMA-senescent HeLa cells. Three independent experiments are shown separately, as the last experiment was processed with a different lot of ELISA kit. The values represent the concentration of IFN β relative to the volume of cultivation medium per 100 000 cells; the numbers shown above the columns represent fold induction relative to control.

'bead array' system, in comparison to IL8, another interleukin produced during oncogene-induced senescence ((Acosta *et al.*, 2008; Kuilman *et al.*, 2008); Figures 3b and c). Increased concentrations of IL6 and IL8 were detected in the culture medium of HeLa cells treated with BrdU + DMA (10 μ M) for 6 days compared with controls. Elevated STAT3 and its Tyr705 phosphorylation indicated the autocrine/paracrine IL6-like activity in BDIS cultures (Figure 3a).

To discriminate the specific contribution of BrdU *versus* DMA to IL6 gene expression (Figure 3d), we followed the dynamics of IL6 mRNA after exposure of HeLa cells to either BrdU or DMA. Compared with rapid elevation of IL6 mRNA after 4 h of DMA treatment (data not shown), which reached the highest levels (~60-fold) on day 6 (Figure 3e), BrdU induced the expression of IL6 only modestly (~threefold) and

with delayed kinetics (after 48 h, Figure 3e). Thus, analogous to IFN β , DMA has a dominant role in the IL6 induction, and the synergy of DMA and BrdU in the induction of IL6 expression correlates with their synergy to induce senescence.

We conclude that the BrdU + DMA-inducible activation of STAT1 is associated with expression and secretion of IFN β and IL6, ligands capable of activating the Janus kinase (JAK)/STAT signaling pathway.

Expression of interferon-stimulated genes is elevated in cancer cells exposed to BrdU and DMA

The enhanced expression and activation of STAT1, and production of IFN β by drug-induced senescent HeLa cells indicated the activity of the interferon-mediated JAK/STAT signaling. To examine whether the

Table 1 List of ISGs with transcripts upregulated by 10 μ M BrdU + DMA

UniGene	Description	Fold ind.	P-value
Hs.517307	Myxovirus (influenza virus) resistance 1	1740.15	0.0003
Hs.632586	Chemokine (C-X-C motif) ligand 10	503.20	0.0005
Hs.20315	Interferon-induced protein with tetratricopeptide repeats 1	170.66	0.0004
Hs.512234	Interleukin 6	151.69	0.0025
Hs.47338	Interferon-induced protein with tetratricopeptide repeats 3	96.67	0.0007
Hs.458485	ISG15 ubiquitin-like modifier	91.77	0.0000
Hs.523847	Interferon, α -inducible protein 6	58.28	0.0004
Hs.163173	Interferon induced with helicase C domain 1	46.37	0.0012
Hs.532634	Interferon, α -inducible protein 27	42.52	0.0007
Hs.458414	Interferon-induced transmembrane protein 1 (9–27)	41.64	0.0006
Hs.401013	Interferon regulatory factor 4	28.25	0.0022
Hs.524760	2',5'-oligoadenylate synthetase 1	25.55	0.0004
Hs.437609	Interferon-induced protein with tetratricopeptide repeats 2	21.41	0.0000
Hs.166120	Interferon regulatory factor 7	12.91	0.0094
Hs.62192	Coagulation factor III (thromboplastin, tissue factor)	11.71	0.0006
Hs.145150	SP110 nuclear body protein	11.31	0.0015
Hs.632790	Interleukin 3 receptor- α	8.66	0.0090
Hs.436061	Interferon regulatory factor 1	7.92	0.0003
Hs.380250	Interferon- γ -inducible protein 16	7.70	0.0041
Hs.82316	Interferon-induced protein 44	5.94	0.0089
Hs.591742	Interleukin 7 receptor	5.92	0.0006
Hs.224645	Pyrin and HIN domain family, member 1	5.45	0.0057

Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; DMA, distamycin A; Fold ind., fold induction of the transcript, relative to control; ISG, interferon-stimulated gene.

Table 2 List of cytokines with transcripts upregulated by 10 μ M BrdU + DMA

UniGene	Description	Fold ind.	P-value
Hs.583348	Inhibin- β A (activin A)	1408.55	0.0008
Hs.624	Interleukin 8	421.68	0.0004
Hs.411311	Interleukin 24	314.08	0.0039
Hs.512234	Interleukin 6 (interferon- β 2)	140.56	0.0023
Hs.501497	CD70 molecule	129.34	0.0003
Hs.170009	Transforming growth factor- α	115.36	0.0000
Hs.272373	Interleukin 20	49.18	0.0028
Hs.473163	Bone morphogenetic protein 7 (osteogenic protein 1)	43.71	0.0003
Hs.126256	Interleukin 1- β	42.22	0.0090
Hs.478275	Tumour necrosis factor (ligand) superfamily, member 10	40.64	0.0000
Hs.591873	Interleukin 7	40.09	0.0010
Hs.1573	Growth differentiation factor 5 (cartilage-derived morphogenetic protein-1)	39.81	0.0008
Hs.241570	Tumour necrosis factor (TNF superfamily, member 2)	38.45	0.0003
Hs.181097	Tumour necrosis factor (ligand) superfamily, member 4	32.33	0.0031
Hs.856	Interferon- γ	27.76	0.0005
Hs.1722	Interleukin 1- α	19.29	0.0028
Hs.370414	Nodal homolog (mouse)	10.85	0.0012
Hs.211238	Interleukin 1 family, member 9	10.02	0.0011
Hs.960	Interleukin 9	8.06	0.0012
Hs.591402	Colony-stimulating factor 1 (macrophage)	5.98	0.0005
Hs.121507	Bone morphogenetic protein 3 (osteogenic)	5.84	0.0088

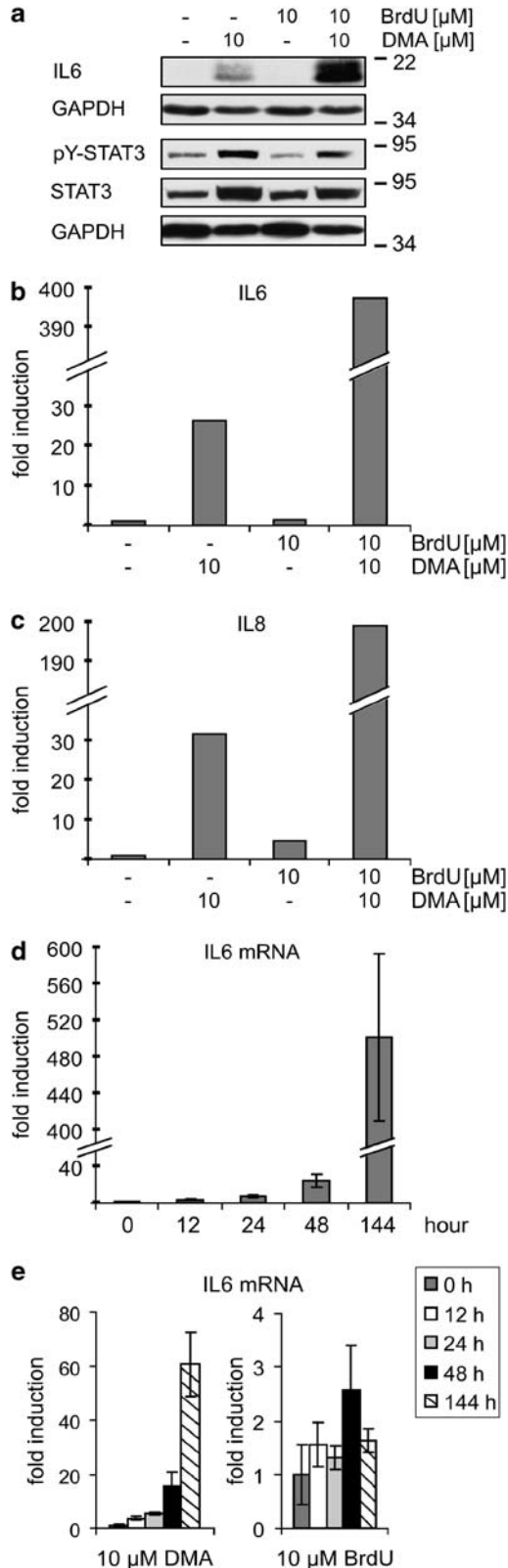
Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; DMA, distamycin A; Fold ind., fold induction of the transcript, relative to control.

transcription targets of the pathway are induced, we compared mRNA levels of 84 interferon-stimulated genes (ISGs) using RT² Profiler PCR Array System ('Interferon and Receptors'), which covers several known targets of IFN type I and II signaling. A total of 22 genes that showed more than fivefold elevation of mRNA in BrdU + DMA-treated HeLa cells (at day 6) compared with controls are listed in Table 1. The most upregulated gene was MX1 (~1700-fold), which is consistent with its strong and exclusive IFN type I inducibility (Ronni *et al.*, 1998). The expression of MX1

was confirmed in time-course experiments at mRNA and protein levels (Supplementary Figures 3a–c). Elevated mRNAs of additional well-known interferon-inducible genes, including chemokine ligand 10 (~500-fold), the ubiquitin-like modifier ISG15 (~90-fold) and 2',5'-oligoadenylate synthetase 1 (~25-fold), were also found (Table 1).

In addition, prolonged expression of IFN β in senescent cells suggested involvement of transcription factors participating in late interferon response (interferon regulatory factors, IRFs), which can maintain

sustained expression of type I IFNs in a positive regulatory loop under conditions such as viral infection (Honda *et al.*, 2005). Indeed, as indicated by 'Interferon RT PCR profiler array' data set, three members of the



IRF family (IRF1, 4 and 7) were upregulated at the mRNA level (Table 1). Among them, IRF1 and IRF7, can be directly involved in stimulation of the IFN β gene (Kim and Maniatis, 1997). We confirmed their expression at the mRNA and protein levels in time-course experiments when BrdU and DMA were used either simultaneously or separately. Gradual increase of IRF1 mRNA to 42-fold and 10-fold elevation (Figure 4a) accompanied by increased IRF1 protein levels (Figures 4c and d) was detected in HeLa cells exposed to 10 μ M BrdU + DMA and 100 μ M BrdU for 6 days, respectively. DMA and BrdU, when used separately, increased the level of IRF1 mRNA by eightfold and twofold after 6 days, respectively. A 120-fold increase of IRF7 mRNA was observed by day 6 using the combined BrdU + DMA, and 22-fold using 100 μ M BrdU alone (Figure 4b). DMA and BrdU (each 10 μ M) used separately increased the level of IRF7 mRNA by 24-fold and 1.6-fold after 6 days, respectively. The increase of IRF7 mRNA levels was also accompanied by the elevation of IRF7 protein (Figures 4c and d). Initial increases (over twofold) of both gene transcripts were detected already by 24-h BrdU + DMA treatment. The onset of IRF1 protein induction by 24-h BrdU + DMA treatment correlated with the induction of IFN β expression (Figure 2b), indicating that IRF1 occurs in early phases of interferon gene induction and, together with IRF7, can participate in the persistent activity of the IFN-JAK/STAT pathway in cells undergoing BDIS. In addition, we found elevated IRF1 in HeLa cells brought to senescence using camptothecin, etoposide (see also Pamment *et al.*, 2002), doxorubicin, APH and HU (data not shown).

Overall, the upregulation of transcripts of several ISGs indicated that BrdU + DMA-induced activity of interferon-JAK/STAT signaling results in corresponding gene expression.

BrdU + DMA-induced expression of ISGs requires JAK1
To prove that activated JAK1/STAT1 signaling induced by BrdU + DMA is indeed instrumental for the induction of ISGs, we knocked down JAK1, the receptor-associated kinase responsible for STAT1 activation, using lentiviral transduction of HeLa cells with vectors carrying short hairpin RNAs (shRNA; Supplementary Materials and methods). Four JAK1-specific

Figure 3 Induction of interleukin-6 (IL6) and IL8 expression during 5-bromo-2'-deoxyuridine plus distamycin A (BrdU + DMA)-evoked senescence. **(a)** Immunoblot detection of IL6 levels, the level of signal transducers and activators of transcription 3 (STAT3) and its Tyr705 phosphorylation status in HeLa cells treated with BrdU and DMA. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as the loading control. Estimation of IL6 **(b)** and IL8 **(c)** in medium conditioned by HeLa cells treated with BrdU and DMA, using the FACS bead array. Quantitative real time reverse transcription PCR quantification of IL6 mRNA levels in HeLa cells exposed to 10 μ M BrdU + 10 μ M DMA **(d)** and the two drugs used separately **(e)**. The values representing average of three independent experiments are shown as fold induction relative to control; error bars represent standard error.

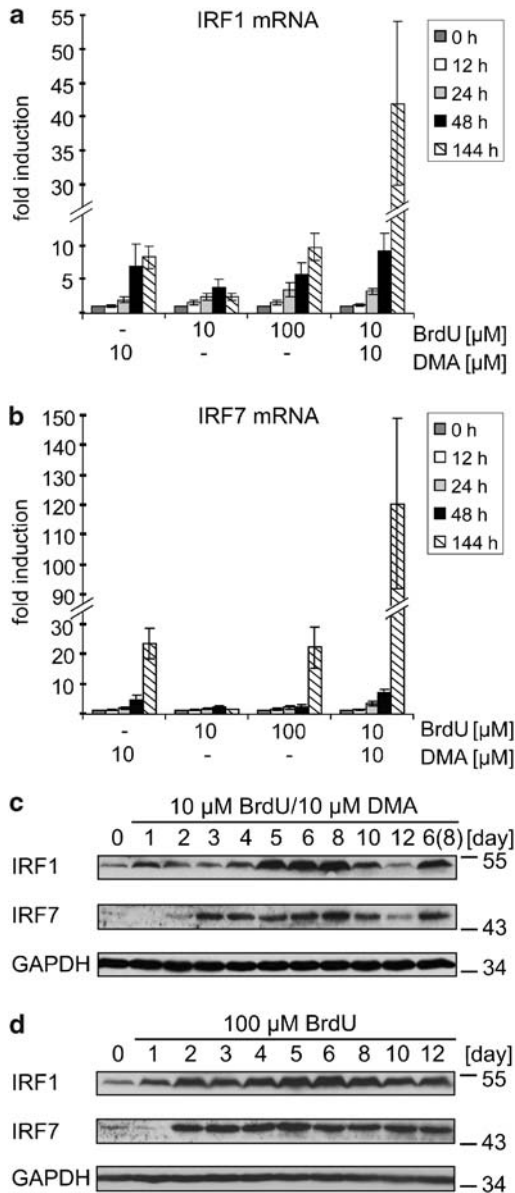


Figure 4 HeLa cells treated with 5-bromo-2'-deoxyuridine plus distamycin A (BrdU + DMA) exhibit increased expression of interferon regulatory factor 1 (IRF1) and IRF7. IRF1 (a) and IRF7 (b) mRNA levels quantified by quantitative reverse transcription PCR in BrdU + DMA-treated HeLa cells. The average values representing three independent experiments are given as fold induction relative to control. Error bars represent standard error. Immunoblot analysis of IRF1 and IRF7 protein levels in HeLa cells treated with 10 μM BrdU plus 10 μM DMA (c) or 100 μM BrdU (d) for various time intervals. Note that elevated levels of IRF1 and IRF7 observed at day 6 remained unchanged for the next 2 days after removal of both drugs from the culture medium (see the last lane in c). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control.

shRNAs showed different efficiency of JAK1 knockdown (Figures 5a and b), which correlated well with the extent of suppression of total and activated STAT1 and several ISGs (that is, IRF1 and MX1, Figure 5c and Supplementary Figure 3c). Thus, the induction of ISGs during development of BDIS depends on active JAK1/STAT1 signaling.

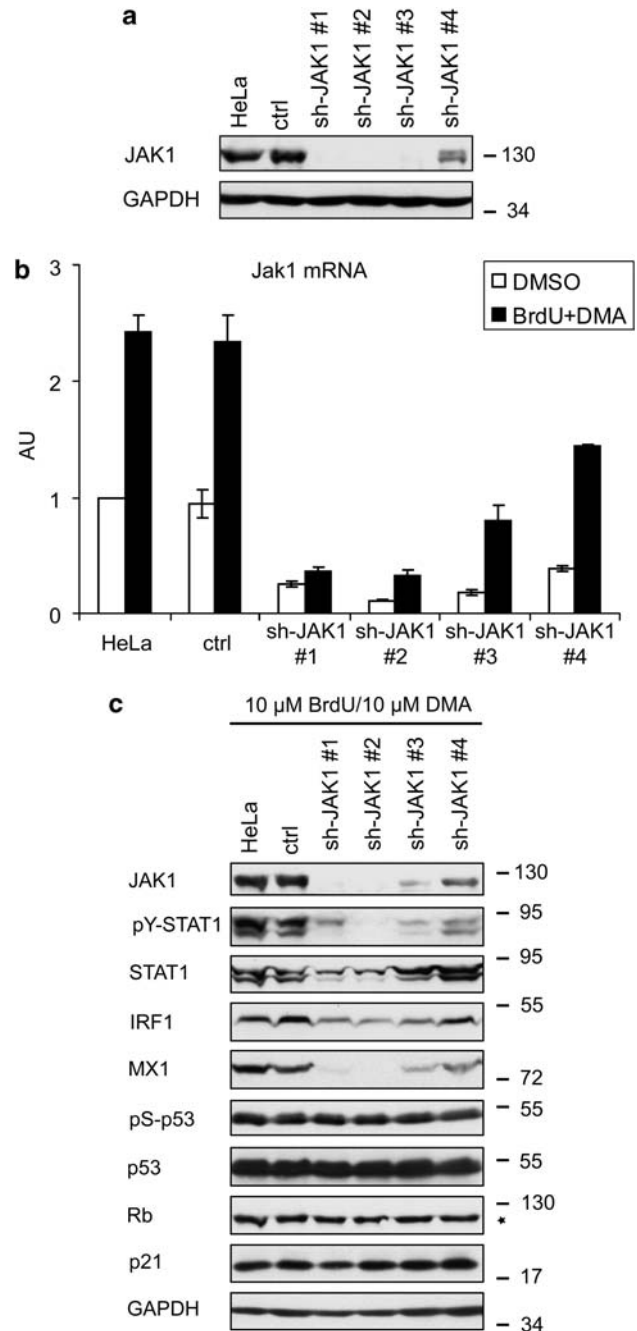


Figure 5 Inhibition of Janus kinase 1 (JAK1) expression aborts signal transducers and activators of transcription 1 (STAT1) activation and expression of interferon-stimulated genes (ISGs), but not DNA damage signaling. (a) Immunoblot analysis of JAK1 expression levels affected by short hairpin RNAs (shRNAs) directed against JAK1 (JAK#1, #2, #3, #4) in HeLa cells. (b) Real-time quantitative reverse transcription PCR estimation of knockdown efficiency of shRNAs directed against JAK1 (shJAK1 #1, #2, #3, #4). The average values representing three independent experiments are given as arbitrary units relative to control. Error bars represent standard error. (c) Immunoblot detection of STAT1 expression and activity, p21, Rb (hypophosphorylated Rb-asterisk), total and serine 15-phosphorylated p53 (pS-p53) and expression of ISGs (IRF1 and MX1) after a 6-day treatment with 5-bromo-2'-deoxyuridine plus distamycin A (BrdU + DMA) in HeLa cells transfected by shRNAs specific for JAK1 (shJAK1 #1, #2, #3, #4) and empty vector (ctrl). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control.

IL6 and JAK1 are not essential for drug-induced DNA damage signaling and BDIS

DNA damage signaling is a feature shared by all types of senescence (Campisi and d'Adda di Fagagna, 2007;

Mallette and Ferbeyre, 2007; Halazonetis et al., 2008), and given the recent reports on oncogene-induced and replicative senescence (Kuilman and Peeper, 2009), complemented by our present data on drug-induced senescence, cytokine/chemokine secretion may represent another universal aspect of cellular senescence. Given that downregulation of IL6 by RNA interference rescued cells from oncogene-induced senescence (Kuilman et al., 2008), we examined whether this causal relationship is also involved in BDIS. Surprisingly, even a substantial shRNA-mediated depletion of IL6 (shIL#1: >90% for protein; Figure 6a and 85% for mRNA; Figure 6b), validated by the loss of potential of the corresponding HeLa-conditioned medium to support IL6-dependent growth of mouse hybridoma B9 cells (>90%; Supplementary Figure 4a), had no effect on receptor-dependent phosphorylation of STAT1 and STAT3, activation of 'senescence markers' (p21, Rb, p53, pS15p53; Figure 6a) and the development of BDIS (Supplementary Figures 4b–i). Furthermore, we found only marginal elevation of CCAAT-enhancer-binding protein β (C/EBP β) mRNA in BDIS (Supplementary Figure 4j), contrary to the robust C/EBP β increase reportedly underlying the IL6-C/EBP β -positive feedback loop that drives BRAF^{E600} oncogene-induced senescence (Kuilman et al., 2008). These results indicate that the IL6-C/EBP β regulatory circuit is not essential for BDIS.

Furthermore, inhibition of JAK1-dependent signaling through JAK1-specific shRNA had no effect on development of BDIS (Supplementary Figures 5a–i). Even inhibition of four kinases of the JAK family (JAK1, JAK2, JAK3, Tyk2) with the specific JAK inhibitor I (2-(1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one), had no effect on activation of p21 and development of senescence itself (Figure 6c; Supplementary Figures 5j–m). Consistently, with preserved senescence, the extent of drug-induced DNA damage signaling, monitored by Ser15-phosphorylated p53 (Figures 5c and 6c), or focus formation of DNA damage markers γ H2AX or 53BP1 in cells

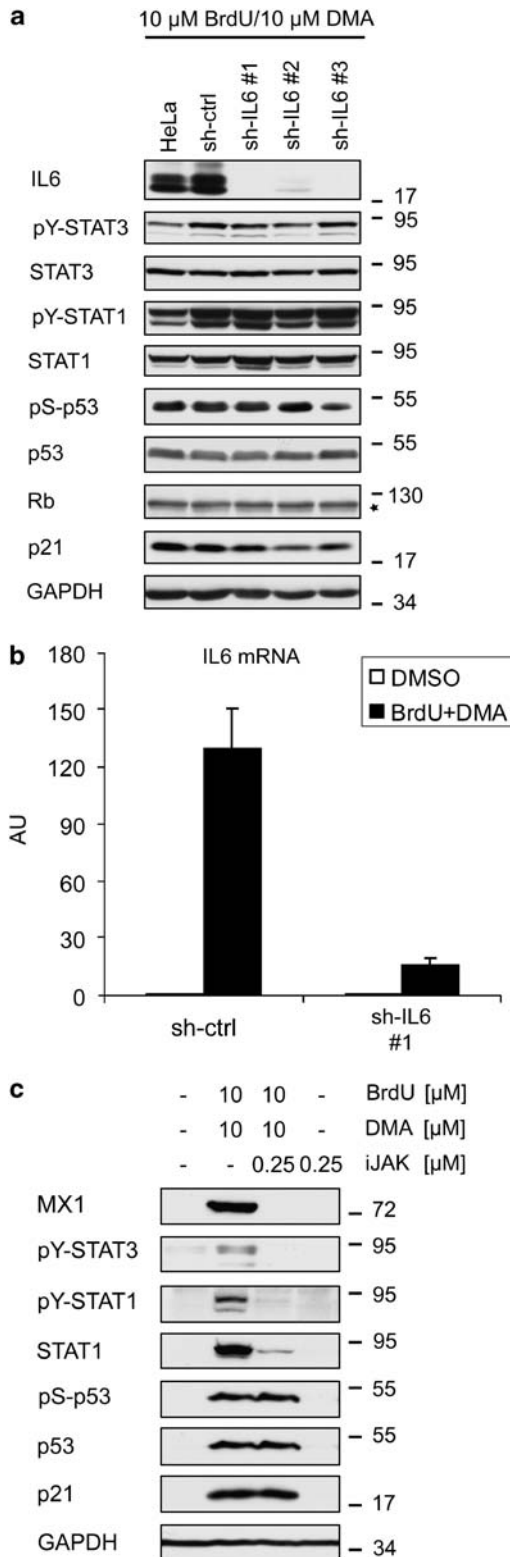


Figure 6 Inhibition of IL6 expression does not affect induction of expression and activation of signal transducers and activators of transcription 3 (STAT3), STAT1 and tumor suppressors after 5-bromo-2'-deoxyuridine plus distamycin A (BrdU + DMA) treatment. **(a)** Immunoblot detection of STAT3 and STAT1 expression and phosphorylation (pY-STAT3 and pY-STAT1), p53 and serine 15-phosphorylated p53 (pS-p53), p21 and Rb (hypophosphorylated Rb—asterisk) after 6 days of treatment with BrdU + DMA (10 μ M) in HeLa cells transfected by short hairpin RNAs (shRNAs) specific for IL6 (shIL6 #1, #2, #3) and nonsense shRNA (sh-ctrl). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control. **(b)** Real-time quantitative reverse transcription PCR estimation of knockdown efficiency of shRNA directed against IL6 (shIL6 #1). The average values representing three independent experiments are given as arbitrary units relative to control. Error bars represent standard error. **(c)** Immunoblot detection of STAT3 and STAT1 phosphorylation (pY-STAT3 and pY-STAT1), MX1, p53 and serine 15-phosphorylated p53 (pS-p53), and p21 after 6 days of treatment with BrdU + DMA (10 μ M) in HeLa cells treated with the chemical Janus kinase inhibitor I (iJAK).

treated by genotoxic drugs (data not shown), remained unaffected after JAK1 knockdown or JAK1 activity inhibition. Importantly, the JAK/STAT signaling was effectively abolished under such conditions, as documented by gross reduction of the most sensitive downstream readout, the MX1 expression (Figures 5c and 6c).

Altogether, these results indicate that IL6-C/EBP β and JAK1-mediated signaling is not required for the drug-induced DNA damage response and senescence.

Discussion

Cellular senescence is a biological phenomenon involved in major pathophysiological processes such as tumorigenesis and aging (Campisi, 2005; Collado *et al.*, 2007). Our present study contributes several findings that help better understand premature senescence induced in tumor cells by diverse genotoxic compounds used in various clinical applications including cancer chemotherapy.

First, we report induction of a secretory phenotype that accompanied such senescence, characterized by prolonged activation of the JAK1/STAT1 signaling pathway and long-term upregulation of several ISGs including tumor suppressors, IRF1, PML, STAT1, mda-7/IL24, and additional cytokines, such as pro-inflammatory IL1 α/β , IL6, IL8, TNFSF7 and TNF α . Our results extend recent broadly analogous findings on replicative senescence and oncogene-induced premature senescence (Acosta *et al.*, 2008; Kuilman *et al.*, 2008), and indicate that such secretory phenotype is shared by all major types of senescence. In addition, the spectrum of induced cytokines/chemokines we observed is significantly broader than those reported for other senescence scenarios, and the involvement of the IFN β -STAT1-ISGs axis seems to be so far unprecedented.

Regulation of IFN-dependent signaling is complex (Shuai and Liu, 2005), yet the gradual onset and long-term persistence of IFN β -JAK/STAT signaling seen in our drug-treated cells indicates engagement of positive regulatory loops reminiscent of those operating in virus-infected cells (Honda *et al.*, 2005). According to such model, virally activated IRFs (IRF7 and IRF3) drive the expression of IFNs, the secretion of which enables the establishment of a positive regulatory loop through autocrine/paracrine mechanisms. The activation of the IFN type I JAK/STAT signaling results in transcriptional induction of IRFs and IFN genes whose products then close and amplify the loop. A similar mechanism might explain the persistent induction of ISGs (including IRF1 and IRF7) also in later phases of BrdU + DMA-induced secretory phenotype reported here. Surprisingly, IFN β , but none of several IFN α species tested here, were induced in drug-exposed cells (Supplementary Table 1), suggesting that expression of IFN type I genes, IFN β and IFN α , is differentially regulated in drug-induced senescence.

Another observation we made is the variability in terms of senescence and cytokine signaling responses, depending on the cancer cell line and genotoxic drug used. The former variability likely reflects the diverse genetic backgrounds in our models, particularly the status of the p53 and RB tumor suppressors whose defects (in MDA-MB-468 and HS913T cells) correlated with rather poor responses compared with p53/RB-proficient U2OS and A549 cells. RB and p53 are critical for proper execution of senescence (Collado *et al.*, 2007; Mallette and Ferbeyre, 2007) and their defects are often accompanied by enhanced constitutive DNA damage (DiTullio *et al.*, 2002), also because such tumors breached the anticancer barrier of DNA damage checkpoints and senescence (Mallette and Ferbeyre, 2007; Halazonetis *et al.*, 2008). The fact that HeLa cells responded relatively well to genotoxic drugs may reflect BrdU/DMA-induced degradation of the papilloma virus oncoproteins E6 and E7 (Suzuki *et al.*, 2001), thereby liberating the endogenous p53 and RB functions, and hence promoting the senescence response.

The latter variability, seen in response to the eight genotoxic compounds and their combinations used here, might be attributable to the distinct modes of drug-DNA interactions, or the types and extent of DNA lesions they cause. In any case, our results on differential abilities of genotoxic drugs to induce complex secretory phenotypes should be kept in mind when applying such compounds in neurological examinations (such as BrdU) and especially chemotherapy (several of the drugs used here).

From the mechanistic point of view, the key phosphorylations of STAT1 detected in our experiments, on Tyr701 and Ser727, are critical for STAT1 translocation into the cell nucleus and maximal transcriptional activation, respectively (van Boxel-Dezaire *et al.*, 2006). STAT1 phosphorylation by JAK/Tyk kinases can be triggered by several cytokines and growth factors, for example, IL6, IL10 and growth hormones, but it is dominantly activated by interferons (Imada and Leonard, 2000), the production of which in response to drugs was revealed by our present study. Recently, IL6 and IL8 have been causally implicated in the induction and maintenance of oncogene-induced senescence (Acosta *et al.*, 2008; Kuilman *et al.*, 2008). Our data confirm and extend these studies, by showing that both IL6 and IL8 are produced in drug-induced senescence also. In contrast to the abolishment of BRAF-oncogene-induced senescence by RNA interference-mediated IL6 depletion (Kuilman *et al.*, 2008), however, neither IL6 nor the JAK1/STAT1 signaling proved to be strictly required for the senescence phenotype induced by genotoxic drugs examined here. The lack of impact is unlikely to be attributable to insufficient knockdown, as IL6 was depleted to undetectable levels (over 90%) and the JAK1 knockdown abolished the downstream events of the JAK/STAT pathway such as MX1 induction. We propose that the secretory phenotype may have a less critical role in the chemically induced premature senescence compared with some other types of cellular senescence. This conclusion is further supported by the

fact that in some drug/cell line combinations reported here, the senescence phenotype occurred in the absence of pronounced JAK/STAT signaling.

We believe that the major driving force behind the genotoxic drug-induced senescence is the activated DNA damage response machinery, evoked by the DNA lesions caused by the drugs we use. Furthermore, DNA damage signaling was not affected by blocking either IL6 or JAK/STAT signaling here (Figures 5, 6 and data not shown), again supporting the notion that DNA damage checkpoints activate p53 and RB, and likely other pathways that impose the proliferation arrest characteristic of senescence. DNA damage signaling also contributes to other types of senescence, including replicative and oncogene-induced cellular senescence (Campisi and d'Adda di Fagagna, 2007; Mallette and Ferbeyre, 2007; Halazonetis *et al.*, 2008), and the severity of cell phenotype depends on the threshold of DNA damage and/or 'oncogenic stress' involved (Bartek *et al.*, 2007). We speculate that the more stringent requirement for cytokine/chemokine signaling in some other types of senescence may reflect the cooperative effects of the relatively modest degree of DNA damage evoked by BRAF oncogene, for example, that requires IL6 signaling to boost the intrinsic anti-proliferative pathways to fully evoke and maintain senescence (Kuilman *et al.*, 2008). In contrast, the extent of DNA damage generated by genotoxic drugs or some other senescence-inducing stimuli, including more potent oncogenes or telomere attrition, might be sufficient to reach the threshold required to trigger senescence.

Redundancy among ligands or signaling modules - in diverse senescence scenarios may also affect the biological outcome of the knockdown experiments. Thus, whereas the dramatic impact of depleting a single cytokine/chemokine ligand or receptor on oncogene-induced senescence (Acosta *et al.*, 2008; Kuilman *et al.*, 2008) implies very little or no redundancy in such secretory network signaling, the lack of impact on IL6 depletion in our experiments can be explained by multiple ligands capable of activating the signaling pathway (Figure 6c), consistent with a broad spectrum of cytokines/chemokines elevated in response to genotoxic drugs. Regardless of their requirement for establishment of senescence, the autocrine/paracrine effects of cytokines in our present experiments contributed to the senescence-associated gene expression pattern, as was shown by induction of many ISGs in senescent cells, and to their gross reduction upon JAK1 knockdown. We hope that our results will inspire further research into drug-induced secretory phenotypes, particularly *in vivo*, in clinical settings in which such drugs are applied for various diagnostic and therapeutic purposes. As the secreted signaling molecules also exert diverse cell non-autonomous effects, such as immunomodulation (Campisi, 2005), and genes such as IFN β or mda-7/IL24 or MX1 that were found elevated in our present study harbor potent tumor suppressor functions (Kaynor *et al.*, 2002; Fisher, 2005; Mushinski *et al.*, 2009), their drug-induced expression may affect the outcome of cancer chemotherapy.

Materials and methods

Chemicals and antibodies

BrdU, DMA, netropsin, Hoechst33258, HU, APH and TriReagent were from Sigma (St Louis, MO, USA) and 5-chloro-2'-deoxyuridine from MPBiomedicals (Eschwege, Germany). The antibodies used are listed in Supplementary Materials and methods.

Cell culture

A549 and U2OS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and HeLa were cultured in the same medium supplemented with 5% fetal bovine serum. Cells were kept at 37 °C under 5% CO₂ atmosphere. For additional cell culture information see Supplementary Materials and methods.

Induction of cellular senescence

On the basis of pilot experiments, 100 μ M BrdU or a combination of 10 μ M BrdU with 10 μ M DMA (dissolved in dimethyl sulfoxide) were chosen for routine administration to reach senescence within 6 days of the treatment. Culture medium with fresh additives was changed every second day. STAT1 phosphorylation and IRF1 and IRF7 expression were estimated on HeLa cells treated with 10 μ M BrdU or 10 μ M DMA for 6 days, followed by thorough medium change and 2-day culture without drugs.

Quantitative real-time reverse transcription PCR

Total RNA was isolated using TriReagent according to the manufacturer's protocol. For IFN β transcripts, purified RNA was treated with 80 U/ml DNase (TURBO DNA-free Kit, Applied Biosystems, Foster City, CA, USA) for 40 min at 37 °C. First strand cDNA was synthesized from 200 ng of RNA with random hexamer primers using TaqMan Reverse Transcription Reagents (Applied Biosystems). Quantitative reverse transcription PCR was performed in ABI Prism 7300 (Applied Biosystems) using SYBR Green I or Power SYBR Green I Master Mix (Applied Biosystems) with the primers shown in Supplementary Materials and methods. The relative cDNA amount was estimated by standard curve, data normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

Determination of cytokine secretion

HeLa cells were treated with 10 μ M BrdU or 10 μ M DMA for 6 days with medium change every second day. The conditioned medium was collected at day 6 and the number of cells per each dish counted. The concentration of IFN β in culture medium was estimated using Human IFN β ELISA kit (PBL Biomedical Laboratories, Piscataway, USA). Absorbance (450 nm) was measured by reader Tecan Sunrise (Grödig, Austria). The concentration of IFN β was derived from standard curve and expressed as picograms per milliliter medium per 100 000 cells. IL6 and IL8 were estimated by 'FACS bead array' using FlowCytomix Human Simplex Kit (BMS8213FF and BMS8204FF, respectively, Bender MedSystems, Wien, Austria) on flow cytometer LSRII (BD Biosciences, San Jose, USA) according to manufacturer's protocol.

Transduction of shRNA

Specific shRNAs were introduced into HeLa cells using lentiviruses. In brief, HEK293T cells were transfected by one shRNA-coding lentiviral expression vector and two lentiviral packaging vectors (pMD2.G, psPAX2, Addgene, www.addgene.org) using calcium phosphate transfection. After 2 days, supernatant was removed and viral particles precipitated for 24 h by

PEG-it virus precipitation solution (System Biosciences, Mountain View, CA, USA). HeLa cells were infected with viral particles by application of supernatant for 48 h. Transduced HeLa cells were selected using puromycin during 6 days. The list of shRNAs used is shown in Supplementary Materials and methods.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)