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Mechanisms of replicative, drug- and oncogene-induced cellular senescence

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Contents

1. Abstrakt

Buňky jsou trvale vystaveny různým formám stresu, který pochází jak z vnitřního, tak z vnějšího prostředí. Buněčná senescence, permanentní zástava buněčného cyklu, představuje fyziologickou odpověď organismu, která zabraňuje proliferaci poškozených buněk. Jedná se o významný mechanismus nádorové suprese, jelikož inaktivace této bariéry v pre-maligních stadiích tumoru vede k rozvoji rakovinného bujení. Na druhou stranu, v dlouhodobé perspektivě se senescence podílí na stárnutí tkáně organismu. Porozumění příčinám a důsledkům senescence nám může poskytnout důležité informace o procesech jako je rakovinné bujení a stárnutí organismu, což může přispět k nalezení nových terapeutických přístupů.

V první části dizertační práce jsme studovali vztah mezi dvěma tumor-suprimujícími signálními dráhami, DDR a ARF, v odpovědi na aktivaci onkogenu. S využitím několika myších modelů, lidských klinických vzorků a buněčných kultur jsme ukázali, že k aktivaci signální dráhy ARF dochází v pozdějším stádiu vývoje nádoru než k aktivaci dráhy DDR. Pro spuštění ARF signalizace je navíc zapotřebí vyšší hladiny onkogenního stresu než pro aktivaci DDR. ARF tedy představuje pozdní a komplementární bariéru nádorové suprese.

Změny chromatinu spojené se senescencí (SAHF) jsou obecně považovány za mechanismus přispívající k zástavě buněčného cyklu v onkogenní a replikativní senescenci. V druhé části této dizertační práce jsme proto analyzovali, do jaké míry jsou změny chromatinu univerzální u různých typů senescence. Zjistili jsme, že některé typy buněčné senescence nejsou doprovázeny změnami chromatinu a ty se tedy vyskytují v závislosti na povaze signálu spouštějícího senescenci a také v závislosti na buněčném typu.

Dále jsme studovali cytokinovou expresi a signalizaci v nádorových buňkách, které byly vystaveny účinku různých genotoxických látek. Naše data ukazují, že senescentní buňky sekretují široké spektrum cytokinů a trvale aktivují JAK/STAT signální dráhu. Tato cytokinová signalizace má autokrinní/parakrinní efekt na genovou expresi, což je relevantní vzhledem k chemoterapii používané při léčbě nádoru.

V poslední části práce poskytujeme nový vhled do současných znalostí o regulaci nádorového supressoru PML v chemickými látkami indukované senescenci. Dle našich výsledků je regulace transkripce PML nezávislá na dráze p53 a je kontrolována signální dráhou JAK/STAT prostřednictvím vazby transkripčního faktoru STAT na element ISRE, jež je lokalizován v promotoru genu PML.

2. Abstract

Cells continuously experience various forms of exogenous and endogenous stress stimuli. Cellular senescence, a state of permanent cell-cycle arrest, is a physiological response that prevents proliferation of damaged cells. It is an important tumor suppressive mechanism, because evading senescence in pre-malignant tumors leads to cancer progression. However, in the long term, senescence can also contribute to tissue aging. Our understanding of the causes and consequences of senescence can provide important insights into processes such as cancer development and aging and may help us design new therapeutic approaches.

In the first part of my thesis, we investigated a relationship between two known tumor suppressor pathways, the DNA Damage Response (DDR) and the Alternative Reading Frame (ARF), in response to oncogenic insults. By using several mouse models, human clinical samples and cell culture models we showed, that ARF activation occurs at a later stage of tumor progression than activation of the DDR. Moreover, ARF activation requires a higher level (threshold) of oncogenic stress than DDR. Therefore, we proposed, that ARF represents a delayed and complementary barrier to tumor progression.

Senescence-associated heterochromatin foci (SAHF) formation, representing pronounced changes in chromatin, has been previously established as a mechanism contributing to maintenance of the senescence phenotype in oncogene-induced and replicative senescence. In the second part of this thesis, we thus analyzed to what extent does SAHF formation represent a universal feature of the senescence program. We concluded that SAHF are dispensable for some types of cellular senescence and occur in an insult- and cell type-dependent manner.

Next, we investigated cytokine expression and signaling in cellular senescence evoked in tumor cells by diverse genotoxic drugs. We found, that senescent cells secrete a broad spectrum of cytokines/chemokines and persistently activate the JAK/STAT signaling pathway. Our data thus suggest autocrine/paracrine effects of cytokine signaling on senescence-associated gene expression, a phenomenon that is likely highly relevant to the outcome of cancer chemotherapy.

Finally, we provide novel insights into our current knowledge about regulation of the tumor suppressor PML in drug-induced senescence. According to our data, transcriptional upregulation of PML is independent of the p53 tumor suppressor pathway and is controlled by JAK/STAT signaling, via binding of the transcription factor STAT to the ISRE element in the PML gene promoter.

3. Introduction

Cellular senescence

Cellular senescence was described more than 40 years ago as a process that limits the growth of normal human cells in culture (Hayflick, 1965). Hayflick and coworkers demonstrated that these cells can proliferate *in vitro* for approximately 55 populations doublings before their proliferative capacity succumbs to proliferation arrest despite the presence of abundant nutrients and mitogens (Adams, 2009; Campisi and d'Adda di Fagagna, 2007). The term cellular senescence therefore denotes a stable and long-term loss of proliferative capacity, despite continued viability and metabolic activity (Kuilman et al., 2010).

Although cellular senescence was first described in the context with excessive rounds of cell division (Hayflick, 1965), it can be caused also by various cellular stresses e.g. oncogene activation or tumor suppressor inactivation (Lin et al., 1998; Serrano et al., 1997; Zhu et al., 1998), oxidative stress (Adams, 2009; Chen and Ames, 1994), DNA-damaging agents such as ionizing radiation (Di Leonardo et al., 1994; Robles and Adami, 1998) and drugs (te Poele et al., 2002; Wang et al., 1998a).

In 1990 it was shown that as human primary fibroblasts are cultured towards the end of their replicative lifespan, telomere length gradually decreases (Harley et al., 1990). Other authors later demonstrated that the activity of telomerase, which is the enzyme counteracting telomere shortening, is high in immortal cells and absent from human somatic cells. At the same time they have shown that human primary fibroblasts can be immortalized by transfection of the catalytic subunit of human telomerase (hTERT) (Bodnar et al., 1998). Telomere attrition was therefore established as a trigger for replicative cellular senescence originally described by Hayflick in 1965 (Hayflick, 1965, 2000).

Oncogene activation is a hallmark of cell transformation and cancer. However, early studies on mutant HRAS led to the discovery that it induces cell cycle arrest when it is introduced alone into primary cells (Land et al., 1983; Serrano et al., 1997). Subsequently, other oncogenes such as BRAF and MEK were shown to cause senescence when expressed as oncogenic versions or overexpressed (Lin et al., 1998; Michaloglou et al., 2005; Zhu et al., 1998). Unlike replicative senescence, oncogene-induced senescence can´t be bypassed by transfection of the catalytic subunit of human telomerase (hTERT), confirming its independence from telomere attrition (Wei et al., 1999).

This initial *in vitro* observation was validated *in vivo* by using mouse models. The conditional gene knock-in mouse model of KRAS was

shown to trigger senescence during the early stages of pancreatic and lung tumorigenesis (Collado et al., 2005). Oncogenic BRAF expression in mouse leads to lung adenomas with features of cellular senescence (Dankort et al., 2007; Dhomen et al., 2009). Transgenic expression of β-catenin induces senescence in murine lymphocytes (Xu et al., 2008). In addition, loss of the tumor suppressor PTEN in mice prostate leads to development of benign lesions with presence of senescent cells (Chen et al., 2005). Importantly, occurrence of senescence during tumor progression has been documented also in human premalignant lesions. Senescent cells were found in human colon adenomas (Bartkova et al., 2006), in benign melanocytic nevi carrying oncogenic mutant BRAF (Michaloglou et al., 2005), in neurofibromas from NF1 mutant patients (Courtois-Cox et al., 2006) and also in early-stages of human prostate cancer (Chen et al., 2005).

Anticancer drugs can usually induce either apoptosis or proliferation arrest, however, senescence can be achieved by using lower drug concentrations than those required to trigger apoptosis (Chang et al., 2002; Rebbaa et al., 2003). Importantly, drug-induced senescence, unlike most other types of premature senescence, can be established in cancer cells (Rebbaa, 2005). Many chemotherapeutic agents have been reported to force cells into senescence. The first group of such drugs represent topoisomerase inhibitors such as campthotecin, etoposide and doxorubicin, which interfere with the ability of topoisomerases to re-ligate DNA (Chang et al., 1999a; Han et al., 2002). Other potent inductors of senescence are alkylating agents, platinum-based drugs causing crosslinking of DNA, and hydroxyurea, a well-known inhibitor of ribonucleotide reductase (Chang et al., 1999a; Yeo et al., 2000).

Several pathways have been shown to play roles in cellular senescence. These include among others the DNA-damage response (DDR), p53-p21 and p16INK4a-RB pathways. Changes in chromatin (SAHF) (Narita et al., 2003) and proteins secreted during senescence (SASP) (Coppe et al., 2008) also contribute to establishment and maintenance of the senescence program.

Ruptures of the DNA backbone can lead to the generation of single-stranded DNA (ssDNA) or DNA double-strand breaks (DSBs). Both structures compromise the structural stability of chromosomes (d'Adda di Fagagna, 2008). Signaling kinases, notably ATR, ATM and DNA-PK, are recruited to the sites of DNA damage and are activated. The recruitment leads to the phosphorylation of Ser-139 of histone H2AX adjacent to the site of DNA damage, which is necessary for focal assembly of checkpoint and DNA repair factors including NBS1, 53BP1 and BRCA1 (Celeste et al., 2002). An increase of local ATR and ATM activity also engage DDR

factors which function far from the site of DNA damage such as transducer kinases CHK1 and CHK2. Both kinases then spread DDR signaling by phosphorylating its substrates throughout the cell (Bekker-Jensen et al., 2006; Lukas et al., 2003).

Importance of chromatin changes in cellular senescence has been discussed for a long time (Ryan and Cristofalo, 1972). A significant mechanistic insight into the role of chromatin in permanent cell cycle arrest maintenance was brought in 2003, when global heterochromatinization of senescent nuclei was described as Senescence-associated heterochromatin foci (SAHF) (Narita et al., 2003).

SAHF can be defined as discrete domains of heterochromatin that form due to large-scale chromosome condensation (Narita et al., 2003). Each SAHF focus is thought to be an individual chromosome (Funayama et al., 2006). Senescence-associated heterochromatin foci can be easily visualized by the DNA dye 4',6-diamidino-2-phenylindole (DAPI). While cycling or quiescent cells show largely homogenous DAPI staining for DNA, senescent cells positive for SAHF display bright positive nuclear foci (Narita et al., 2003).

SAHF, which were initially described in replicative and oncogene-induced senescence in human fibroblasts, contain several common markers of heterochromatin such as methylated histone H3 on Lys 9 (H3K9Me), hypoacetylated histones and Heterochromatin Protein 1 (HP1) (Narita et al., 2003). At the same time, depletion of a linker histone H1 and enrichment of a histone variant macroH2A or HMGA are considered to represent SAHF-specific marks (Funayama et al., 2006; Narita et al., 2006). Several pathways have been demonstrated to contribute to the SAHF formation.

Senescence-Associated Secretory Phenotype and JAK/STAT signaling pathway

Senescent cells show pronounced changes in gene expression of many secreted proteins such as metalloproteinases or cytokines (Bavik et al., 2006; Coppe et al., 2008; Kuilman et al., 2008; Shelton et al., 1999; Wajapeyee et al., 2008). The first clue of changes in secretome of senescent cells was revealed by microarray analyses of replicatively senescent fibroblasts (Shelton et al., 1999). Since that it has been shown that the secretory phenotype occurs in different cell types such as human melanocytes, human breast epithelial cells, human endothelial cells and mouse lymphoma cells, and it has been termed senescence-associated secretory phenotype (SASP) (Chien et al., 2011; Coppe et al., 2008; Kuilman et al., 2008; Maier et al., 1990).

Cytokines are key mediators of the immune response and play important roles also in processes such as autoimmune diseases, cancer and cellular senescence (Aringer et al., 1999; Dinarello, 2007; Kuilman et al., 2008). They are produced by many different types of cells and can be subdivided into several subgroups including interferons, interleukins, mesenchymal growth factors, tumor necrosis factors, adipokines and chemokines (Dinarello, 2007).

Cytokines bind to their cognate receptor and trigger intracellular signaling through Janus tyrosine kinases (JAKs) and their transcription factors STATs (signal transducers and activators of transcription). There are four members of JAKs family (JAK1, JAK2, JAK3 and TYK2) and seven members of STAT known. Cytokine signaling via the JAK/STAT pathway impacts and changes gene expression (Jatiani et al., 2010).

Particulary, ligand binding to cytokine receptor localized at the cytoplasmatic membrane induces receptor dimerization/oligomerization. Subsequently, JAKs undergo autophosphorylation and this activation of JAKs results in phosphorylation of receptors on tyrosine residues. STATs are recruited via their SH2 domains to activated receptors and become phosphorylated by Janus kinases. Phosphorylated STATs can dimerize and their translocation from cytoplasm to the nucleus facilitates activation or suppression of target genes (Baker et al., 2007).

Promyelocytic leukemia protein (PML)

The promyelocytic leukemia (PML) tumor-suppressor was initially identified in acute promyelocytic leukemia (APL), where PML gene (on chromosome 15) can be subject to fusion with the retinoic acid receptor α (RARα) gene (on chromosome 17). The chromosomal translocation t(15;17) results in proeduction of the PML-RARα fusion protein, which plays a driving role in acute promyelocytic leukemia (APL) (de The et al., 1990; Melnick and Licht, 1999).

To date, several reports showed, that PML expression and formation of PML NBs increase upon oncogenic expression and DNA damage (Ferbeyre et al., 2000; Pearson and Pelicci, 2001; Scaglioni et al., 2012). In oncogene-induced senescence, PML transcription is controlled by the tumor suppressor protein p53, through p53 response element in the PML gene (de Stanchina et al., 2004).

PML promoter also contains IFN-response elements (ISREs) and IFNγ activated sites (GAS), which are responsive to interferons (Stadler et al., 1995).

The promyelocytic leukemia (PML) tumor-suppressor has been implicated in regulation of heterochromatin changes in oncogene-induced and replicative senescence. Several SAHF (Senescence-Associated Heterochromatin Foci) components transiently colocalize with PML-NBs as cells approach senescence (Ye et al., 2007; Zhang et al., 2005). Specifically, the chromatin regulator HIRA and heterochromatin protein HP1 enter into PML-NBs, prior to deposition of HP1 into SAHF. HIRA relocalization is considered to be one of the earliest events in the senescence program, which precedes appearance of other markers of senescence such as cell cycle exit, changes in morphology, SA-β-gal activity and importantly SAHF formation. It has been also shown, that recruitment of HIRA to PML bodies correlates with incorporation of histone macroH2A (a transcription-silencing histone H2A variant) into chromatin and that ectopic expression of HIRA accelerates SAHF formation (Zhang et al., 2005).

A strong link between PML bodies and SAHF formation was supported in two ways. First, expressions of mutant HIRA, which blocks localization of endogenous HIRA into PML-NBs, abolished SAHF formation. Next, formation of SAHF was also blocked once PML-NBs are disrupted by the PML-RARα fusion protein, a hybrid protein with altered functions (Ye et al., 2007). Overall, the PML bodies seem to play a catalytic role in assembly and modification of proteins prior to their translocation to chromatin and formation of SAHF (Zhang et al., 2005). However, once cells approach senescence, PML-NBs and SAHF form distinct nuclear structures (Vernier et al., 2011).

4. Aims of the study

The main objective of this study is to provide novel insights into several aspects of cellular senescence, which are important from not only mechanistic and conceptual points of view, but also highly relevant to cancer therapy. The aims are sub-divided into four parts, each represented by a separate study, dealing with the following issues and open questions:

- **Publication 1:** Activation of DDR and ARF pathways in response to oncogenes
  - Are DDR and ARF triggered concurrently or at different stages of cancer progression in response to oncogenic signaling?
  - Is there any difference in activation thresholds between DDR and ARF?
  - What is the relative contribution of DDR and ARF tumor suppressor pathways during oncogene-induced senescence?

- **Publication 2:** Insights into chromatin changes during cellular senescence
  - Is Senescence-associated heterochromatin foci formation (SAHF) a universal feature of cellular senescence?
  - What is the correlation between SAHF formation and $p16^{INK4a}$ pathway activation?
  - Is detection of SAHF applicable as a marker for studies *in vivo*?

- **Publication 3:** Role(s) of the senescence-associated secretory phenotype (SASP) in drug-induced senescence
  - Is cellular senescence induced by diverse genotoxic compounds accompanied by protein secretion analogous to replicative and oncogene-induced senescence?
  - If yes, what is the spectrum of induced secreted proteins?
  - Which specific signaling pathways are activated?

- **Publication 4:** Regulation of the tumor suppressor protein PML in cellular senescence
  - What is the molecular mechanism of PML induction in genotoxic drug-induced senescence?

5. Material and methods

- Allelic imbalance analysis
- BrdU proliferation assay
- ChIP assay
- DNA copy number and gene expression analysis
- ELISA
- Flow cytometry
- Gel retardation assay
- Immunohistochemistry
- Indirect immunofluorescence
- Luciferase reporter assay
- Preparation of stable cell lines using lentivirus transduction
- Quantitative real time RT-PCR (qRT-PCR)
- SDS-PAGE and western blotting
- Senescence associated-β-galactosidase assay
- Statistical analysis

6. List of publications

Publication 1: Evangelou, K., Bartkova, J., Kotsinas, A., Pateras, I.S., Liontos, M., Velimezi, G., **Kosar, M.**, Liloglou, T., Trougakos, I.P., Dyrskjot, L., Andersen, C., Papaioannou, M., Drosos, Y., Papafotiou, G., Hodny, Z., Sosa-Pineda, B., Wu, X.R., Klinakis, A., Orntoft, T., Lukas, J., Bartek, J., Gorgoulis V.G. (2013). The DNA damage checkpoint precedes activation of ARF in response to escalating oncogenic stress during tumorigenesis. Cell Death Differ. 20, 1485-1497.
Impact factor: 8.371

Publication 2: **Kosar, M.**, Bartkova, J., Hubackova, S., Hodny, Z., Lukas, J., Bartek, J. (2011). Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16 (ink4a). Cell Cycle 10:3, 457-468.
Impact factor: 5.243

Publication 3: Novakova, Z., Hubackova, S., **Kosar, M.**, Janderova-Rossmeislova, L., Dobrovolna, J., Vasicova, P., Vancurova, M., Horejsi, Z., Hozak, P., Bartek, J., Hodny, Z. (2010). Cytokine expression and signaling in drug-induced cellular senescence. Oncogene 29, 273-284.
Impact factor: 7.357

Publication 4: Hubackova, S., Novakova, Z., Krejcikova, K., **Kosar, M.**, Dobrovolna, J., Duskova, P., Hanzlikova, H., Vancurova, M., Barath, P., Bartek, J., Hodny, Z. (2010). Regulation of the PML tumor suppressor in drug-induced senescence of human normal and cancer cells by JAK/STAT-mediated signaling. Cell Cycle 9:15, 3085-3099.
Impact factor: 5.243

7. Results and discussion

1. *The DNA damage checkpoint precedes activation of ARF in response to escalating oncogenic stress during tumorigenesis*

Oncogene activation can lead to premature senescence or apoptosis via activation of the p53 pathway (Collado and Serrano, 2010; Halazonetis et al., 2008). Although the DNA damage response (DDR) and alternative reading frame (ARF) pathways are known to trigger p53 during oncogenic stress (Bartkova et al., 2005; Bartkova et al., 2006; Gorgoulis et al., 2005; Sherr, 2012; Sherr and Weber, 2000), precise mechanisms of any functional interplay between these two anti-cancer barriers remain unclear.

To shed light on this issue, in the first part of this study we asked whether DDR and ARF are triggered concurrently or at different stages of cancer development. *In vivo* analysis of several experimental mouse models showed that DDR markers are expressed earlier than ARF during progression of premalignant lesions to malignancy. Analysis of human samples of head and neck, skin, urinary bladder and pancreatic lesions during cancer progression lead us to the same conclusion. Overall, results in the first part of our study showed that DDR activation preceded ARF induction and ARF thus likely provides a delayed checkpoint-barrier during cancer development.

Next, we asked why ARF signaling is not active during initial steps of cancer development. To answer this question we employed human bronchial epithelial cells (HBECs) and BJ fibroblast models *in vitro*, into which various oncogens were sequentially introduced. While DDR activation was evident upon expression of single oncogene (e.g. a mutant, p16-insensitive CDK4) in HBECs, ARF activation required an additional oncogenic event, here provided by mutant KRAS. Experiments with BJ fibroblasts led to the same conclusion, because even though DDR was activated upon expression of one oncogene (β- catenin or HRAS, respectively), ARF was induced only upon combined (concomitant) transformation by β-catenin together with HRAS. Importantly, even prolonged expression of the single oncogene (HRAS) in BJ fibroblasts was not sufficient to induce ARF activation, in contrast to efficient induction of the DDR. These data indicate that ARF provide a less-sensitive tumor suppressor barrier than DDR. At the same time, late upregulation of ARF, which was observed during cancer development *in vivo*, could be a consequence of accumulating oncogenic insults.

To examine the relative 'biological' contributions of DDR versus ARF pathways under conditions of increased oncogenic load, we first co-

expressed β-catenin and HRAS in BJ fibroblasts. Next, we silenced either DDR or AFR or both pathways at the same time. While inhibition of single pathway led to the partial bypass of cellular senescence, concomitant silencing of ARF and DDR was more potent.

Overall, our results suggest that ARF provides complementary and delayed barrier against the risk of malignant transformation and, when activated, it acts in concert with the DDR.

A potential crosstalk between the DDR and ARF pathways has been recently described in two studies. First of them focused on the role of MRE11 in oncogene induced senescence (Gupta et al., 2013). MRE11 is a known sensor of DNA double-strand breaks, which plays a role in the activation of the DDR (Stracker and Petrini, 2011). Authors showed that in response to oncogene expression, the MRE11 complex mediates the G2 arrest in mouse mammary hyperplasia via DDR activation. Impairment of MRE11 functions, and thus DDR, lead to progression of hyperplasias into aggressive and metastatic breast cancers. Importantly, hypomorphic mutations in the MRE11 complex components caused oncogene-dependent activation of the ARF pathway and selection for Ink4a-ARF locus inactivation was observed at later stages of tumor development (Gupta et al., 2013).

The other recent study also suggests a close interplay between the DNA damage response and ARF pathways (Velimezi et al., 2013). ATM, the prominent DNA damage response kinase, can suppress ARF protein levels and activity in oncogene-transformed and cancer cells. The authors proposed that ATM activates protein phosphatase 1 (PP1), which antagonizes NEK2-dependent phosphorylation of nucleophosmin (NPM). PP1-dependent dephosphorylation of NPM weakens the interaction between ARF and NPM, and the liberated ARF was subsequently susceptible to proteasome degradation. On the contrary, ATM inhibition enhanced ARF levels and promoted p53-dependent tumor suppressor functions of ARF. Data from human clinical samples showed that reduced expression of ATM at advanced stages of tumor progression correlated with increased ARF protein levels in lung carcinomas. In conclusion, this publication supports the role of ARF as a secondary anti-cancer barrier, which can be particularly activated upon loss of ATM (Velimezi et al., 2013).

2. Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16 (ink4a)

Senescence-associated foci (SAHF) were first described by Narita and Lowe, who observed SAHF formation upon oncogenic RAS activation in human diploid fibroblasts IMR90 (Narita et al., 2003). This pioneering work was later followed by several other studies, which were mostly focused on the mechanism of SAHF formation in oncogene-induced senescence (Ye et al., 2007; Zhang et al., 2005).

Since the previous publications used only few strains of human fibroblasts, data regarding SAHF formation in a broader spectrum of cell types, especially the cancer highly relevant human epithelial cells, were lacking. At the same time there was lack of information about any significance of SAHF formation in senescence caused by diverse stimuli. Since a universal marker of cellular senescence has not been identified yet, another important issue was the potential applicability of SAHF as a biomarker of senescence *in vivo*.

In order to extend the studies published previously and to answer the above-mentioned open questions we decided to systematically compare the ability to form SAHF in various types of normal human cells exposed to different senescence-inducing stimuli. Moreover, we have analyzed SAHF formation in premalignant and malignant human lesions.

Senescence-associated heterochromatin foci formation accompanied by silencing genes that drive cell cycle progression has been considered to be critical for induction of senescence (Narita et al., 2003). However, our data implies that SAHF are not a common feature of the senescence program and that there is a strong variation in SAHF formation depending on cell type and senescence stimulus. Although SAHF formation upon oncogenic RAS expression was obvious in all tested cell strains (BJ, MRC5 and normal human keratinocytes), only MRC5 were able to form SAHF in response to chemotherapeutic drugs (hydroxyurea, etoposide, doxorubicin), excessive rounds of cell division or bacterial intoxication (HdCDT). Importantly, markers of cellular senescence (SA-β-gal expression, diminished BrdU incorporation, DDR activation) were present in all strains upon all senescence insults. Therefore this part of our study lead us to the conclusion that some types of cellular senescence can be established in the absence of SAHF formation.

This conceptually important observation was recently confirmed also by several other authors. First of them noticed that although BJ fibroblasts became senescent following telomere shortening, exposure to

ionizing radiation or H2O2 treatment, detectable SAHF were missing (Di Micco et al., 2011). Variable and partly cell-type specific compaction of DNA was observed also by Contrepois (Contrepois et al., 2012), who studied the interplay between regulation of deacetylation of histone H4-K16Ac and heterochromatin assembly during senescence.

Finally, a recent study that focused on evaluation of markers and their development and specifity in replicative senescence, revealed the lack of SAHF formation in BJ fibroblasts in contrast to MRC5 (Schauble et al., 2012).

The second key aspect of our study was the critical role of the p16INK4a-RB pathway in SAHF formation. Importance of the p16INK4a-RB pathway in SAHF development upon oncogene activation had been proposed previously (Narita et al., 2003; Ye et al., 2007). We have systematically tested any correlation between SAHF and p16INK4a induction in MRC5 and BJ fibroblasts, undergoing senescence induced by different stimuli. Biochemical analysis of p16INK4a expression in BJ cells revealed increased p16INK4a expression only upon oncogenic RAS activation, the only type of cellular senescence where we detected SAHF. In contrast, MRC5 showed increased p16INK4a levels in all types of senescence. Moreover, there was a perfect correlation between the level of p16INK4a expression and SAHF formation. These results are in line with the proposed role of the p16INK4a-RB axis in SAHF development (Narita et al., 2003).

Finally, our data are also relevant to the biological role of senescence as anti-cancer barrier (Bartek et al., 2007; Campisi, 2005). Enhanced heterochromatinization in clinical samples of premalignant human lesions detected by H3K9Me and HP1 was reported previously (Bartkova et al., 2006). Whether heterochromatinization is accompanied by the presence of DNA/DAPI-defined SAHF in such human samples has not been investigated before. Although examination of paraffin sections of formalin-fixed pellets of senescent BJ cells with oncogenic RAS expression revealed presence of DNA/DAPI-defined SAHF (used as a positive control scenatio), we were unable to detect any clear DNA/DAPI-defined SAHF on tissue sections of normal human colon, adenomas and carcinomas. At the same time heterochromatin markers H3K9Me and HP1 were present in these tissue samples. Thus, heterochromatin domains observed in human tissues appear to be different from the DNA/DAPI-defined SAHF which can be detected in cell culture models. Although precise mechanistic understanding of differences in heterochromatin biology in premalignant human lesions and cell culture models will require more studies, it is clear that

identification of senescent cells through detection of the DNA/DAPI-defined SAHF may not be applicable in human lesions.

3. Cytokine expression and signaling in drug-induced cellular senescence

One of the features of cellular senescence is the striking change in gene expression of many secreted proteins (Bavik et al., 2006; Wajapeyee et al., 2008). To date, more than fifty cytokines have been found to be secreted by senescent cells (Davalos et al., 2010). Senescence-associated secretory phenotype (SASP) has been previously investigated mainly in replicative and oncogene-induced senescence (Acosta et al., 2008; Kuilman et al., 2008; Shelton et al., 1999). In order to extend our current knowledge about the role of SASP in premature senescence, we decided to examine cytokine secretion in cells treated by various genotoxic drugs, including those used in cancer chemotherapy.

In agreement with previously published data (Michishita et al., 1999; Suzuki et al., 2002), long-term administration of BrdU and DMA led to premature senescence of cancer cells. Interestingly, we found that treatment with those chemicals persistently activates the IFNβ-JAK/STAT signaling pathway. This pathway was previously shown to be transiently evoked in response to virus infection, where infection leads to secretion of interferons which contributes to establishment of a positive regulatory loop via an autocrine/paracrine mechanism (Honda et al., 2005). Beside prolonged activation of the JAK/STAT signaling pathway, we found up-regulated expression of many other secreted proteins such as ISG15, PML, MX1, IL-6, IL-8, IL-10 and IL-24. Our results inspired two conceptually important conclusions. First, the spectrum of proteins secreted by senescent cells is much broader than published previously (Acosta et al., 2008; Kuilman et al., 2008). Second, secretion of diverse proteins is probably common in all major types of cellular senescence.

It has been shown, that cytokines IL-6 and IL-8 are directly involved in the establishment and maintenance of oncogene-induced senescence and depletion of both cytokines abolished the senescence phenotype (Acosta et al., 2008; Kuilman et al., 2008). Whereas we did find IL-6 and IL-8 expressed in genotoxic drugs-induced senescence, in our hands depletion of IL-6 was unable to overcome senescence. Moreover, markers of senescence such as pS15p53, p21 and RB, were present even after inhibition of the JAK/STAT signaling pathway through depletion of kinases of JAK family or treatment with a JAK specific inhibitor. Thus, in

contrast to the previously published role of IL-6 in oncogene-induced senescence, our data indicate that IL-6 and JAK mediated signaling are not essential for maintenance of drug-induced senescence. It has been shown, that DNA damage signaling is essential for maintenance of diverse types of senescence (Campisi and d'Adda di Fagagna, 2007; Halazonetis et al., 2008). At the same time, a certain threshold level of DNA damage is necessary for activation of cellular senescence (Bartek et al., 2007). Therefore, we speculate, that the observed different outcomes of IL-6 depletion in drug-induced senescence versus senescence evoked by the BRAF oncogene (Kuilman et al., 2008), could be explain by different DNA damage thresholds in such distinct biological settings. Particularly, if DNA damage level is lower in BRAF-induced senescence, one possibility is, that cells need to boost the cell cycle arrest using other pathways such as IL-6 signaling. In contrast, the high level of DNA damage after drug administration is sufficient to maintain genotoxic-stress induced senescence without IL-6 signaling.

Taken together, findings presented in this study extend our knowledge about proteins secreted during genotoxic drug-induced cellular senescence. Because many of the secreted proteins that we found are known to play roles in the immune system and tumor suppression, our data are highly relevant to cancer therapies.

4. Regulation of the PML tumor suppressor in drug-induced senescence of human normal and cancer cells by JAK/STAT-mediated signaling

Critical roles of the tumor suppressor PML in several processes related to tumor suppression have been described extensively (Chen et al., 2012). Beside the other PML functions, such as regulation of apoptosis or cell migration, there are several reports showing that PML induction and formation of PML NBs is a common feature of replicative, oncogene- or bacterial-induced senescence (Blazkova et al., 2010; Ferbeyre et al., 2000; Janderova-Rossmeislova et al., 2007).

Our previous work, where we reported induction of a senescence-associated secretory phenotype upon cell exposure to diverse genotoxic compounds, revealed prolonged activation of JAK/STAT signaling and upregulation of numerous important tumor suppressors including PML (Novakova et al., 2010). At the same time, it is known that the PML promoter contains sequences (ISREs, GAS) which can bind STAT transcription factors (Stadler et al., 1995). Therefore we decided to

investigate whether PML expression is directly regulated by the JAK/STAT signaling pathway in drug-induced senescence.

First, we showed that treatment with diverse genotoxic drugs (BrdU, Thymidine, Distamicin, Etoposide, Aphidicoli and Campthotecin) leads to higher numbers of PML NBs in normal fibroblasts and also in human cancer cell lines. Multiplication of PML NBs was accompanied by increases in both PML protein and also mRNA levels. At the same time, there was no difference in PML protein stability as revealed by treatment with cycloheximide, a known inhibitor of de novo protein synthesis. Taken together, those data showed that the observed increase of PML NBs can be attributed to enhanced PML expression after treatment with genotoxic drugs.

Next we asked, whether expression of PML in drug-induced senescence requires JAK/STAT signaling. Indeed, chemically or genetically down-regulated signaling via JAK led to decreased levels of PML mRNA in drug-induced senescent cells. Two STAT binding sites (ISREs and GAS) in the PML gene promoter were published previously (Stadler et al., 1995), therefore we asked, which (if any) of them is required for transcription of PML after genotoxic stress. Data obtained by gel retardation assay revealed that the STAT binding that contributes to PML expression requires ISRE-mediated transcription. This result was also confirmed by a reporter assay with the PML promoter containing or lacking the ISRE element, respectively.

It has been published that the PML promoter contains, besides the STAT binding sites, also a p53 response element and, furthermore PML transcription can be controlled by p53 in oncogene-induced senescence (de Stanchina et al., 1998; Stadler et al., 1995). Therefore, we decided to investigate whether PML expression via JAK/STAT signaling relies on transcriptional activity of p53. For that, we used two different systems. First, human cell lines HCT-116 with wild-type or deleted p53 were treated with thymidine in the presence or absence of a JAK inhibitor. In this setup, PML expression was inhibited by JAK inhibition in both cell lines, showing that indeed JAK/STAT signaling is crucial for regulation of PML transcription upon genotoxic stress, regardless of p53 expression. Second, we used the U2OS cell line conditionally expressing (or not) the dominant-negative tumor suppressor p53, which were published previously (Thullberg et al., 2000). Because also in this case there was no significant difference in levels of PML expression, we conclude, that in this case, transcriptional activation of PML upon genotoxic stress is not dependent on p53.

Many types of cellular senescence are maintained by persistent DNA damage signaling, which can reflect irreparability of some types of

DNA lesions (von Zglinicki et al., 2004). Previous studies showed that PML NBs co-localized with sites of persistent DNA damage foci after ionizing radiation (Carbone et al., 2002; Xu et al., 2003). These types of lesions are usually positive for markers of DNA double strand breaks such as γH2AX and 53BP1 (Rogakou et al., 1998; Schultz et al., 2000). Although in our experiments cells were treated with genotoxic drugs instead of ionizing-radiation, consistently with previous studies PML NBs also co-localized with persistent DNA damage foci.

The exact purpose for the co-localization of PML NBs with persistent DNA damage foci needs to be investigated. One possible scenario is that PML NBs, known sites of localization of chromatin-modifying proteins such as HP1 (Zhang et al., 2005) and HDACs (Wu et al., 2001), can promote changes in chromatin architecture surrounding DSB to impact persistent DNA damage signaling. In support of this idea, recently published work showed that PML NBs can represent the site of chromatin remodeling that regulates transcriptional activity of the E2F promoters through their recruitment into the close proximity of high concentrations of histone deacetylases (HDACs) (Vernier et al., 2011).

Overall, our study provides novel mechanistic insights into regulation of the tumor suppressor PML in cellular senescence caused by several genotoxic drugs used in clinical chemotherapy. These emerging links between cytokine signaling and PML can be important especially in the light of the fact that, unlike other forms of cellular senescence, drug-induced senescence can be evoked even in tumor cells and hence be exploited therapeutically.

8. Conclusions

The major aim of this work was to contribute to better understanding of several key aspects of cellular senescence. From a broader perspective, our data are highly relevant for complex processes such as development of cancer, aging, and response to clinical chemotherapy. We hope that our findings will positively stimulate further research in this exciting area of biomedicine.

- **Publication 1:** Activation of DDR and ARF pathways in response to oncogenes
  - DDR activation occurs at earlier stage of tumor progression than ARF
  - DDR requires a lower threshold of oncogenic stress for its activation
  - ARF thus represents a delayed and complementary barrier to tumor progression

- **Publication 2:** Insights into chromatin changes during cellular senescence
  - Senescence-associated heterochromatin foci formation (SAHF) is not a universal feature of cellular senescence
  - SAHF occur in insult- and cell type-dependent manner
  - SAHF formation correlates well with $p16^{INK4a}$ accumulation
  - SAHF are not markers applicable to studies *in vivo*

- **Publication 3:** Role of senescence-associated secretory phenotype (SASP) in drug- induced senescence
  - Senescence induced by diverse genotoxic compounds is accompanied by secretion of a broad spectrum of secreted proteins including pro-inflammatory cytokines
  - Autocrine/paracrine signaling mediated by persistent activation of the JAK/STAT pathway regulates gene expression in senescent cells and contributes to maintenance of cytokine signaling

- **Publication 4:** Regulation of the tumor suppressor protein PML in drug-induced senescence
  - PML is regulated at the transcriptional level by JAK/STAT signaling via binding of the transcription factor STAT to the ISRE element in the PML gene promoter

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