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**DNA damage and signalling pathways in cellular senescence**

Ph.D. Thesis

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# 1. Abstract

Organisms such as mammals need tissue renewal as an important process for maintenance of their viability. Because proliferation is essential also for tumourigenesis, cells need tumour-suppressor mechanisms to protect organism against cancer. Cellular senescence, the permanent state of cell-cycle arrest, features one of these intrinsic barriers against tumourigenesis after DNA damage and understanding of this process may lead to finding of novel therapeutic targets and to optimization of chemotherapy for patients with cancer.

In the first part of the PhD thesis, we investigated an activation of JAK/STAT signalling pathway in drug-induced senescence. We used genotoxic drugs like aphidicolin, camptothecine, 5-bromo-2'-doxyuridin, etoposide or thymidine to induce premature senescence in normal and cancer cells. All these chemicals were able to persistently activate JAK/STAT signalling in monitored cells. Activation of STATs was accompanied with up-regulation of expression of interferon-stimulated genes (ISGs), such as MX1, IRF1, IRF7 and PML. Since IRF1 and IRF7 can be directly involved in stimulation of the IFN genes, we show activated expression as well as secretion of IFN $\beta$  and IFN $\gamma$ , but not IFN $\alpha$  in drug-induced senescent cells. Furthermore, an inhibition of JAK1 as a major kinase of STAT activation led to suppression of STAT activation and expression of ISGs, supporting a dominant role of JAK1 in activation of interferon response in drug-induced senescent cells. Besides interferons, we found significantly elevated expression and secretion of several other cytokines, like pro-inflammatory IL1, IL6, IL8 and IL10 or TNF $\alpha$ . Because JAK/STAT is activated through cytokines (mainly by interferons and IL6), our results suggest a positive feedback loop between JAK/STAT prolonged induction and cytokine secretion.

Promyelocytic leukaemia protein (PML) is one of the well known tumour suppressors upregulated in almost all forms of cellular senescence. It is an essential component of nuclear structures known as PML NB nuclear bodies (NBs) whose accumulation occurs in response to stress. These bodies represent sites where activation of many regulators of cell cycle progression including p53 and Rb proceeds. As we showed, PML NB also colocalize with sites of DNA double strand breaks detected by accumulation of  $\gamma$ H2AX (modified form of histone H2AX marking sites of DNA strand breaks) or 53BP1. In drug-induced senescence, we found that upregulation of PML, a building component of PML NBs belonging to interferon-stimulated

genes, is mediated by JAK/STAT pathway and not by p53, as knockdown of JAK kinase family abolished activation of JAK/STAT pathways as well as PML gene expression and formation of PML NBs in senescent cells in contrast to knockdown of p53, which has no effect on level of PML mRNA and protein. Next we found that transcription of PML is controlled through the Interferon Stimulated Response Element (ISRE) located on its proximal promoter, which is known binding site for STATs, and deletion of this sequence suppressed the transcription efficiency of PML promoter.

The number of PML NBs differs in various cell types cultivated under unperturbed conditions. Therefore we analyzed, whether PML transcription is regulated by JAK/STAT signalling pathway also on its basal level. Interestingly, we found that the number of PML NBs and the levels of PML transcript and protein correlate with activity of STAT3, but not STAT1 or STAT5 in three model cell lines selected for low (U2OS), middle (HeLa) and high PML expression (BJ). Similar correlation was observed between secretion of IL6 - a major activator of STAT3 signalling pathway - and PML NBs levels. Knock-down of STAT3 by specific siRNA as well as a depletion of IL6 from medium by neutralizing antibodies down-regulate PML expression. We found that IL6 alone is able to activate PML transcription in exposed cells. Using chromatin immunoprecipitation, we proved direct binding of STAT3 but not STAT5 to ISRE of PML gene regulatory region.

STAT3 is a main effector activated by IL6, but recent works described IL6-dependent activation of NFkB, a transcription regulator of set of genes involved in immune response, inflammation, proliferation and DNA damage response through IL6/PI3K/Akt signalling pathway. Interestingly, we found that downregulation of NEMO, a molecule important for NFkB activation, also led to downregulation of PML transcription after IL6 treatment.

It is reported that PML NBs play an important role as a docking site for many proteins involved in DNA damage signalling and senescence including those involved in formation of so called „senescence-associated heterochromatin foci“ (SAHF), such as HIRA, HP1 and Rb. While HP1 is well known family of proteins playing role in heterochromatinization, Rb was recently found as the protein involved in formation of SAHF after binding to PML NBs. Elevation of PML NBs was found in all types of senescence, therefore we investigated a presence of SAHF in different types of senescence. We found that all tested cell types formed SAHF under the condition of

oncogene-induces senescence but not after treatment with etoposide, doxorubicin, hydroxyurea or bacterial genotoxins where their formation was cell type specific and correlated with accumulation of cyclin-dependent kinase inhibitor p16INK4A in cells. These results indicate that SAHFs are not a common feature of cellular senescence. Since SAHF formation was described to play the role in silencing of genes important for maintenance of cell proliferation, our results show that these structures are not necessary for induction of senescence but if they are present they support development of senescence through activation/stabilization of p16INK4A/Rb pathway.

## 2. Introduction

### DNA damage response in cellular senescence

The cell division is essential for survival and development of multicellular organism. Since major mission of the cell division cycle is a precise duplication of genome followed by equal dividing of chromosome copies into subsequent cell generations, cells disposed of biochemical pathways called checkpoints playing crucial role in protection of cell against cancer development after DNA damage due to control of its proliferation[1]. DNA may be damaged by many different insults and on different levels. Independently of type of lesion, all DNA damage activates cellular checkpoints which slow or arrest cell-cycle progression, thereby allowing time for appropriate repair mechanisms to correct genetic lesions before they are passed onto the next generation of daughter cells[2]. There are two main pathways recognizing sites of DNA damage. MRE11, RAD50 and NBS1 to the sites of double strand breaks and form MRN complex, which recruit and activate sensor transducing kinases ATM (ataxia telangiectasia mutated kinase)[3]. The second important kinase ATR (ATM and Rad3 related kinase) is recruit to the site of damage by is binding protein ATRIP, which recognize RPA bound on single strand DNA breaks[4]. When activated, ATM and ATR phosphorylate signal transducer checkpoint kinases Chk1 and Chk2, which in turn activates a cascade of cell cycle regulators like p53, p21, CDC25, p16 or Rb hypophosphorylation and blocks the cell in G1-phase to allow the cell to repair damage. When the DNA lesion is irreparable persistent DNA damage signalling followed with inhibition of CDKs via activated checkpoints results in permanent cell cycle arrest or cell death[5]. Thus p53/p21 and p16/Rb present two crucial regulators of senescence and apoptosis.

More than half a century ago, L. Hayflick described a process that is limiting the proliferation of normal human cells in culture and termed it cellular senescence[6]. Later, cellular senescence was shown to be not only the consequence of aging but also a component of tumour suppressive mechanisms[7] and can be induced not only by telomere shortening (replicative senescence), as described Hayflick, but also by oncogene activation, various DNA damaging stress stimuli like ionizing or ultraviolet radiation or different chemicals often used

also in chemotherapy in so called premature oncogene-, stress- or drug induced senescence, respectively[8, 9].

Although cellular senescence and apoptosis represent the barrier against tumourigenesis they differ in manner how they do this. While apoptosis kills and eliminates cancer cells from organism, senescence irreversibly blocks progress throughout the cell cycle. The border between apoptosis and senescence is not strict and many activated proteins and pathways are shared by both tumour barriers[10]. Several recent studies provide some insight into the mechanism of this decision, where for example DNA binding ability of p53 may be one of these regulatory mechanisms[11].

In general, senescence appears to be beneficial, because it protects organisms from tumourigenesis by induction of proliferation arrest in damaged cells as well as was shown that senescent cells are able to recruit immune cells by secreted pro-inflammatory cytokines to execute the clearance of senescent cells from tumour and hereby prevent its rapid growth[12]. However, senescent cells lost ability of regeneration. Therefore from point of homeostasis maintenance, senescence can be deleterious because it contributes to reduction of tissue renewal and function by disruption of tissue structure and changed functional status of senescent cells[13].

Note however, there is no specific marker common to all types of senescence or type of cells or cell line. Senescent phenotype is therefore characterized as a complex of changes represented by altered cellular morphology, increased activity of senescence-associated beta-galactosidase (SA B-gal), increased formation of senescence-associated heterochromatin foci (SAHF) and promyelocytic leukaemia protein nuclear bodies (PML NBs), permanent DNA damage foci, and an inflammatory secretome as will be discussed later[14, 15].

## **JAK/STAT signalling pathway**

JAK/STAT pathway is one of the important signalling activated by cytokines that execute their biological functions essential for organism survival. In mammals, four JAKs (Janus family tyrosine kinases) and seven STATs (signal transducer and activator of transcription) members were identified, which are activated after binding of cytokine into specific receptor resulting in



its dimerization and tyrosine phosphorylation. This provides a docking site for JAK kinase, which in turn activates STATs and allows them to go to the nucleus[16].

The most important role of JAK/STAT signalling was found during viral infections, where IFNs, are involved in numerous immune interactions as inducers, regulators, and effectors of both innate and adaptive antiviral mechanisms as demonstrated by loss of responsiveness to both types of IFN and enhanced susceptibility to bacterial and viral pathogens in STAT1 or IFNAR receptor knock-out mice[17, 18].

Except viral infections, JAK/STAT signalling pathway was observed to be activated also in senescent cells. Few years ago, Moiseeva et al. described that constitutive exposure of cells to interferon beta induces cell cycle arrest due to activation of p53 in dependence on increased levels of ROS[19]. To show a direct role of STATs in senescence development, Ferbeyre et al. published that constitutively activated STAT5A (ca-STAT5A) induces the Rb pathway by down-regulating Myc protein and increasing CDK inhibitor p15INK4b[20].

STAT3 is a member of the STAT family that was found to control a key signalling pathway in the development of many malignant diseases. Its role was observed in the regulation of apoptosis, cell differentiation, proliferation, angiogenesis, metastasis and immune responses that candidates this protein for cancer therapy. Most of the major human malignancies like leukaemia, melanoma or breast and prostate carcinomas show elevated levels of constitutively activated STAT3 as well as elevation of STAT3-regulated gene expression. For many cancers, poor prognosis is associated with elevated levels of activated STAT3[21].

STAT3 is a cytoplasmic protein, which after translocation into the nucleus acts as a key transcription factor of many genes. Few years ago David Levy and his colleagues brought evidence of STAT3 localized into mitochondria and its role in regulation of mitochondrial metabolic function after RAS transformation[22]. During search for its localization in mitochondria, STAT3 was found to bind to complex I of the respiratory chain. Since mitochondrial respiration produces reactive oxygen species, recent studies indicate that the inhibition of STAT3 increases mitochondrial membrane potential, production of ROS and causes DNA damage[23].

## **Promyelocytic leukaemia protein**

PML protein was originally discovered in patients with acute promyelocytic leukaemia (APL), which represents a highly malignant subtype of acute myeloid leukaemia (AML) comprising about 10% of all AML cases. Translocation of PML and retinoic acid receptor alpha (RARalpha) gene was found in almost 95% of APL patients, which results in inability of PML to assemble into microspeckles known as PML nuclear bodies (PML NBs) and inhibits its function[24]. As a result of alternative splicing of PML gene, we can recognize seven PML isoforms (I – VII) utilizing nine exons. Six isoforms (isoform I-VI) are localized in nucleus whereas PML isoform VII is in cytoplasm due to lack of exon 6 containing nuclear localization signal[25]. Two important STAT binding sites were identified in PML gene regulatory region: IFN-stimulated response element (ISRE, at position of 595-628 bp from transcription start) and IFNgamma-activated site (GAS, at position 342-390 from transcription start)[26]. Next to the STAT pathway, p53 was found to be required for induction of PML expression in response to oncogenic RAS, that indicates PML participation in p53-mediated processes including cell cycle arrest or apoptosis[27]. Several posttranslational modifications, such as phosphorylation[28], ubiquitylation[29] and sumoylation[30] was found on PML protein.

PML protein is involved in many cellular processes like gene transcription, proteasomal degradation, viral pathogenicity, DNA damage repair, cellular senescence, tumour suppression or apoptosis, although depletion of this protein is not lethal and PML knockout mice are viable but with increase incidence of tumours[31]. PML NBs, spherical and macromolecular structures resulting from assembly of multiple cellular proteins (with PML as an essential protein for its formation) into distinct bodies, were found to provide a storage site allowing the cell to maintain proper levels of various nuclear factors as well as post-translational modification of several proteins like p53, Rb or several other proteins included in DNA damage response takes place here[32]. Secondly, colocalization of PML NBs with 53BP1 and gammaH2AX in the later phase, but not early after DNA damage was observed, speculation about necessity of PML for formation of chronic DNA lesions was expressed[33].

PML was found to be increased in all types of senescence. In search, whether PML alone is able to induce senescence, only PML isoform IV was found to arrest cell cycle because of

increase p53 phosphorylation and its stabilization and increase pool of hypophosphorylated Rb[34]. PML is a strong tumour suppressor, thus it is not surprising that its levels in many types of human tumours are downregulated. Rather than inhibition of its expression, degradation in proteasome was observed. PML overexpression in cancer cells induces cell cycle arrest and decreases tumorigenicity of these cells[35]. Except regulation of tumorigenicity, PML play important role in viral defence on several levels. PML was found to inhibits ability of the virus to activate expression of genes necessary for its replication, block its integration into host genome, block binding of DNA polymerase on viral genome or target viral proteins for degradation by nuclear structure called clastosome with proteosomal activity[36].

## **Cytokine signalling**

Recent data have expanded the concept that inflammation is a critical component of tumour progression. Many cancers arise from site of infection, inflammation and chronic cytokine signalling. Organism produces a wide range of signals in response to tissue injury trying to heal it. Chemotactic cytokines produced by infected cells recruit cells of immune system (mainly neutrophils and monocytes) into site of damage. Persisting signalling of this cytokines, especially pro-inflammatory TNFalpha, IL6, IL8 and IL1beta was found to be responsible for chronic diseases and cancer development[37]. In senescent cells, cessation of cell proliferation and senescence-associated changes in gene expression, are accompanied with a robust increase in the mRNA levels and secretion of numerous cytokines, chemokines, growth factors and proteases. This phenomenon was termed senescence-associated secretory phenotype (SASP) or senescence messaging secretome (SMS). The SASP, or at least selected components of the SASP, play important roles in autocrine and paracrine signalling[38].

As the SASP results primarily from genomic damage response, one of its beneficial functions is to communicate with cells of the immune system through secretion of pro-inflammatory cytokines to signal that there is a damaged cell and potential risk of tumour development[39]. SASP may also function in regeneration of a tissue after damage, as induction of senescence has been observed in sites of skin wounding or liver damage. Senescence in skin fibroblasts appeared to be important for limiting fibrosis during damage repair[40]. Finally, the

SASP includes factors that help maintain the tumour suppressive growth arrest[13]. On the other hand, increased numbers of senescent cells lead to decline or inhibition of body organ functions in an age-dependent manner. A connection has been found between the accumulation of senescent cells and age-related diseases. It has been found that not only the local microenvironment pathology, but also a variety of chronic degenerative diseases as well as cancer can be induced by circulating pro-inflammatory cytokines like IL6[41].

Recent observations indicate that not only direct damage but also long-term exhibition of stress-induced cytokines released from senescent or damaged cells trigger activation of DNA damage response in neighbouring cells and may induce growth arrest and senescence on basis of ROS induction of DNA breaks formation, which mean potential risk for normal cells to become a part of carcinogenic spreading and clarify mechanism, how senescent cells are able to amplify their number, which results in organ dysfunction and development of age-related diseases[42].

Thus, at present, senescent cells and their secretory phenotype seem to be both beneficial (tumour suppression) and deleterious (age-related diseases) depending on the circumstances and their ability to communicate with surrounding cells including cells of the immune system.

### **3. Aims of the study**

The main aim of the study was to evaluate the role of signalling pathways in mechanism of cellular senescence.

The specific aims of this study were:

- To characterize cytokine expression during cellular senescence
- To investigate the role of cytokine signalling pathways activated during cellular senescence in regulation of expression of tumour suppressors associated with DNA damage and cellular senescence
- To analyze the role of JAK/STAT pathway in regulation of expression of PML in cellular senescence

### **4. Material and methods**

Cell culture

Immunoblotting

Immunofluorescence

shRNA and siRNA transfection

$\beta$ -galactosidase assay

Quantitative real time RT-PCR

Nuclear extraction

Plasmid cloning and transfection

Determination of cytokine secretion by FACS beads

Chromatin Immunoprecipitation (ChIP)

B9 proliferation assay

Luciferase assay

## 5. Discussion

### *1. Cytokine expression and signalling in drug-induced cellular senescence.*

Cellular senescence, a barrier against tumourigenesis, is accompanied with many processes like irreversible cell cycle arrest and activation of tumour suppressor genes. In this study we provide an insight into the activation of JAK/STAT signalling pathway in premature senescence induced in tumour cells by various types of drugs used in chemotherapy, which may help to understand this phenotype and its clinical relevance.

IFN-JAK/STAT signalling pathway is known to be an important part of defence mechanisms against viral infection, where the activation of this pathway protects against viral replication in the cell and activates cells of the immune system. In contrast to viral infection, where JAK/STAT1 pathway is activated only transiently, we found a prolonged activation of JAK/STAT1 signalling (for several days) in cells induced to premature senescence by different drugs. The key phosphorylation of STAT1 on tyrosine 701 and serine 727, which are necessary for STAT1 translocation to nucleus and its transcription activity, were detected. After STAT1 activation, the induction of its downstream targets including tumour suppressors like IRF1, IRF7 (interferon regulated factor 1 and 7), STAT1 and MX1 (myxovirus 1 protein) was observed. IRFs were described to induce expression of interferons and consistent with this we showed for the first time the activation of expression and secretion of IFN $\beta$  and IFN $\gamma$  in senescent cells. Surprisingly, there were no changes in eight IFN $\alpha$  isoforms measured, which indicates different regulation of IFN $\alpha$  and IFN $\beta$  genes in drug-induced senescence. Recent studies implicate the interferon regulatory factors IRF-3 and IRF-7 as key common activators of the IFN $\alpha$  and IFN $\beta$  genes. The discrepancies in expression of IFN $\alpha$  and IFN $\beta$  observed in drug-induced senescent cells may be explained by different binding preference of IRF-3 and IRF-7 to promoters of individual interferon genes [43]. Long term persistence of IFN-JAK/STAT signalling indicates an engagement of a positive autocrine/paracrine regulatory loop involving IRFs, which is reminiscent of that operating in virus-infected cells[44]. In addition to interferons, elevated levels of more than two dozens of cytokines including IL6, IL8, IL24, TNF family, IL1 $\beta$  and IL20 were

found in drug-induced senescent cells. Note, the spectrum of cytokines detected is significantly broader than those reported for other types of senescence.

JAK family kinases play a crucial role in signal transmission from activated receptors. Downregulation of JAK1, a kinase capable to activate all STAT family members, as well as chemical inhibition of all JAK kinases with a specific inhibitor, blocked STAT1 phosphorylation and abolished subsequent downstream events such as induction of IRF1, MX1 and STAT1 itself both on mRNA and protein levels, but had no effect on activation of p53/p21 and p16/Rb pathways, two critical mechanisms in establishment of senescence, SA-beta-gal induction and development of senescent phenotype in general after drug treatment. These results were underscored by experiments where cellular senescence was induced in cell lines without JAK/STAT activation and thus indicate that JAK/STAT pathways are not prerequisite for drug-induced senescence in tumour cells.

At the time we have had been finishing this study, two studies reported quite unexpected findings that IL6 and IL8 are directly implicated in the induction and maintenance of oncogene-induced senescence[45, 46]. Although we confirmed the production of these cytokines also in drug-induced senescent cells, in contrast to study of Kuilman et al., knockdown of IL6 (> 90% efficient) was not able to prevent chemically-induced senescence, as well as activation of p53 and hypophosphorylation of Rb. Despite of IL6 knockdown, activation of STAT3 remained unchanged in drug-induced senescent cells, which indicates more complex mechanism of JAK/STAT3 activation in drug-induced senescence - likely due to a redundant role of multiple ligands capable of activation of this signalling pathway (such as interferons [47]).

In this work we show that activation of DNA damage signalling and induction of senescence in response to various drugs activates but is not dependent on JAK/STAT signalling. This indicates that this form of senescence differs from oncogene-induced senescence which induction and maintenance appears to be dependent solely on IL6 production. Nevertheless, the activation of JAK/STAT1 and STAT3 pathways in senescent cells plays an important role in induction of senescence-associated secretory phenotype (SASP), especially in induction of expression and secretion of interferons, which play an important role in immune cell activation and recruitment to the place of inflammation, and expression of important tumour suppressors IRF1, IRF7 and PML. We speculate that the production of cytokines and chemokines by

senescent cells contributes to their clearance by innate immunity, as is indicated in recent studies. Notably, the capability of SaOS cells, which lack functional p53 and Rb, to undergo drug-induced senescence, suggests involvement of other (i.e. p53/pRb-independent) senescence-inducing mechanism. One such candidate may be based on IRF1/p21Waf1/cip1 axis, which is activated by interferons in drug-induced senescence. If confirmed experimentally, this will support the importance of cytokine signalling in development of this form of senescence.

## ***2. Regulation of the PML tumour suppressor in drug-induced senescence of human normal and cancer cells by JAK/STAT-mediated signalling.***

The induction of PML and its accumulation in nuclear compartment known as PML nuclear bodies are among the well known markers found in all type of senescence and decreased levels of this tumour suppressor were found in more than two thirds of human cancer types [48]. In the previous work discussed we described the activation of JAK/STAT1 and JAK/STAT3 signalling pathways in drug-induced senescent cells. Since PML belongs to the group of interferon stimulated genes[26], we tested whether PML is directly induced by activated JAK/STAT pathway in senescent cells. We found the elevated levels of PML protein, PML NBs and also PML mRNA in tumour cell lines and human primary fibroblasts after treatment with various types of drugs. Experiments with cycloheximide, which blocks de novo protein synthesis, showed that elevation of PML NB and PML protein in senescent cells is accompanied by the increase of PML transcript rather than PML stabilization at protein level. During these experiments, significantly longer PML half-life was observed in comparison with data published by Scaglioni et al. (>24 vs. 3 hrs.). Since they measured PML stability only in virally transformed HEK293 and mouse 3T3 cells and analyzed only one PML isoform, we suggest that PML protein turnover may vary among cell types, the nature of stress and PML isoforms[49].

PML NBs were described to be a docking site for many proteins involved in regulation of the cell cycle and DNA damage response signalling. Colocalization of PML NBs with DNA damage foci not immediately, but in later phases after DNA damage induction [50] suggests their role in formation of persistent DNA damage foci, which may block cell cycle recovery possibly due to irreparability of specific type of DNA lesions. In agreement with these studies, we also



found colocalization of PML NBs with sites of long-term persisting DNA damage foci (positive for modified histone H2AX and other markers of DNA double strand breaks such as 53BP1), but not with acute DNA damage foci formed within first 12 hours after treatment by different DNA damaging drugs. Therefore, formation of PML NBs close to the sites of DNA damage might remark problematic lesions without chance to be repaired, which results in persistent activation of DNA damage response taking place partially in PML NBs. Although Rodier et al. shows that inhibition of PML NBs formation has no effect on persistent DNA damage foci induction[33], different role of PML NBs in DNA damage response is still not excluded.

Stadler et al. described binding of STAT1 and STAT2 on PML promoter after interferon stimulation[26]. In our work, we observed that downregulation of JAK1 kinase abrogates STAT1 activation and induction of its downstream targets. Here we used a specific inhibitor to block all four JAK kinases and demonstrated decreased PML expression in drug-induced senescent cells. Moreover, a decrease in the level of JAK1 kinase induced with a specific siRNA results again in downregulation of PML expression indicating an important role of this specific kinase in regulation of PML transcription. Since inhibition of JAK signalling abrogates PML expression only partially (50%), additional mechanisms such as the regulation by NFkB pathway, which is known to be induced in senescent cells and the putative NFkB or Sp1 binding motifs were found on PML promoter, may be involved in PML gene regulation. Beside interferons, JAK1 kinase may be activated by many other cytokines which we found expressed after genotoxic stress, for example IL6, IL8 and IL10. Therefore, there might be some redundancy in activation of JAK/STAT signalling, which can explain the differences between various forms of senescence, as mentioned above. Which cytokine(s) produced by senescent cells is involved in activation of JAK/STAT signalling and expression of PML is a task for future studies.

PML promoter contains several STAT binding sites. Stadler et al. identified two specific DNA binding elements, ISRE at position 628-595 and GAS at position 390-342 relative to transcription start site, which mediate induction of PML expression after interferon treatment[26]. Our results using gel retardation assay demonstrated the involvement of ISRE, but not GAS element, in regulation of PML transcription in drug-induced senescent cells. Reporter assay with wild type proximal PML promoter (1.44 kb) and PML promoter lacking the ISRE element verified these results, which strongly indicate the necessity of ISRE element in

chemically induced PML transcription. It was described recently that STAT-IRF1 heterodimer binds on ISRE element of LMP2 promoter[51]. Since the overexpression of IRF1 leads to elevation of PML transcript[52] and since we detected elevated level of IRF1 dependent on JAK/STAT activation after genotoxic stress (discussed above), there is a possibility that PML is regulated also by this transcription factor. However, this hypothesis needs further experimental clarification.

The activation of DNA damage response in cellular senescence includes stabilization of p53. Since p53 was described to regulate PML expression in mouse embryonal fibroblasts after RAS activation[27], we tested whether p53 may regulate PML also in our model of drug-induced senescence. For this experiment, we used p53 wild type and p53 knock-out HCT116 cell lines. Both cell lines were able to respond with induction of both PML mRNA and protein and development of senescent phenotype after drug treatment indicating that p53 is not necessary for increased PML expression (and senescence per se) under these conditions. When we inhibited the activity of kinases JAK with the specific inhibitor after drug treatment, we clearly showed a decrease of PML protein level as well as transcript in both cell lines providing the evidence the JAK/STAT pathway is involved in PML regulation in senescent cells. These results were verified using U2OS cells with tetracycline-regulated expression of dominant negative form p53 (U2OS DN-p53 tet-off system[53]) where we also observed no differences in PML expression after DNA damaging drug treatment between wild type and dominant negative p53-expressing cells. There is a question, whether and how the activation of DDR after genotoxic stress may contributes to JAK/STAT activation and PML transcription. Beside the activation of cytokine expression through activation of p38MAPK/NFkB/IL1, IL6, IL8 pathway[39], an activation of c-ABL kinase via ATM[54] and subsequent STAT1 phosphorylation can be also involved.

Our results provide novel mechanistic insight into the regulation of PML mRNA expression during drug-induced genotoxic stress and point to the significance of autocrine/paracrine stimulation in development of senescent phenotype, as was described in our previous study.

### ***3. Interleukin 6 signalling regulates PML gene expression in human normal and cancer cells.***

In our previous studies we found the activation of JAK/STAT signalling pathways in drug-induced prematurely senescent cells and its role in induction of PML gene expression in these cells. As already mentioned, PML protein is the well described tumour suppressor, but regulation of its expression is poorly understood. Two signalling pathways, JAK/STAT and p53 were reported to participate in regulation of PML gene transcription after interferon stimulation and oncogenic RAS activation[26, 27]. Both of these studies described transient elevation of PML mRNA and protein under the stress conditions, but none has addressed the mechanism of constitutive expression of this antiviral and tumour suppressor under unperturbed conditions.

As described previously, various cell lines and primary cells express different levels of PML protein under unperturbed conditions in vitro[55]. We selected three human cell types that feature low (U2OS), medium (HeLa) and high (BJ, at population doublings <35) numbers of PML nuclear bodies. By qRT-PCR, we confirmed a correlation between PML protein level and PML mRNA in these three types of cells. Intriguingly, similar to drug-induced senescent cells, we found the direct involvement of cytokine autocrine/paracrine signalling in regulation of basal levels of PML. We observed increased PML transcription and protein induction in U2OS cells treated with conditioned medium from BJ cells (i. e. cells with high level of basal PML). As we observed no changes in the level of DNA damage response (measured by activation of p53 and phosphorylation of histone H2AX as a marker of double strand breaks) and activity of stress signalling (determined as the activation of p38MAPK), we ruled out any effect of activated p53 on PML transcription. When we analyzed the activity of the STAT pathways (STAT1/2, STAT3, STAT5) in our three selected cell types, we found direct correlation of activated STAT3, but not STAT1 and STAT5 with PML NBs, PML mRNA and protein levels. Using specific cytokine FACS beads assay, we determined conformity in the amount of STAT3 activating ligand IL6 secreted into culture media. IL6 added into culture medium of U2OS or HeLa cells induced PML transcription and the increase of number of PML NBs. Depletion of IL6 from medium using a specific neutralizing antibody or knock-down of STAT3 by siRNA decreased STAT3 active form, PML NBs, PML mRNA and protein level in BJ cells. Kinetic study of STAT3 activation and PML expression after IL6 treatment showed delayed responsiveness of U2OS cells, where

phosphorylation of STAT3 on tyrosine 705 was observed not before 48 hours after treatment and correlated with delayed increase of total STAT3, which indicates the correlation between constitutive level of STAT3 and the dynamics of response to IL6. In comparison with U2OS cells, the response of HeLa cells, which possess higher basal level of STAT3 protein, was fast approaching the rate characteristic for JAK/STAT signalling. Since STAT3 protein is regulated by itself in positive feedback, we suggest that U2OS cells with low level of STAT3 need more time to express it, which likely generates a delay observed in response of PML gene expression to IL6 treatment.

Deletion of PML ISRE element at position (-595/-628) led to a decrease of luciferase reporter gene activity in cells exposed to interferon alpha or beta as well as in cells exposed to various DNA damaging drugs inducing premature senescence. We confirmed the importance of this element also for the regulation of basal PML transcription. We proved the direct binding of STAT3 on PML promoter using chromatin immunoprecipitation. This is not so unexpected, as all members of the STAT family recognize similar consensus binding site. In fact, the PML ISRE element resembles STAT3 consensus better than that of STAT1[56]. Taking together, our data indicate that IL6 controls PML gene expression under unperturbed conditions via JAK/STAT3 signalling and activated STAT3 binds directly to PML gene promoter.

In addition to JAK/STAT3 activation, binding of IL6 on its receptor triggers also the PI3K/Akt pathway[57] which controls NFkB activation. Indeed, the active form of Akt kinase correlated with secreted IL6 in all three cell lines tested. Knock-down of NEMO, the gamma subunit of IKK kinase and a key component of NFkB activation, resulted in suppression of PML gene transcription and protein induction, but simultaneously led to decrease in IL6 expression and secretion and STAT3 activation. Consistent with the known ability of NFkB to control the expression of IL6 gene, the effect of NEMO knock-down on PML expression and down-regulation of NFkB activity was abolished by recombinant IL6, which suggests indirect role of NFkB in PML regulation via modulation of IL6 production. Combined knock-down of NEMO and STAT3 did not result in additive or synergistic effect on PML expression which support our conclusion that NFkB acts in series with STAT3 signalling rather than in parallel (Fig. 5I). Despite these results we still cannot exclude the possibility that PML may be partially regulated by NFkB, as several putative NFkB binding site are present on PML promoter. As was discussed

in previous work[51], the involvement of IRF1 in regulation of PML basal level is another alternative . Since IRF1 gene is known target of NFkB, the decreased level of PML transcription after IL6 or NEMO depletion may be partially influenced by inhibition of IRF1 expression, as is shown on figure 5H.

In summary, the novel finding of this study is that in addition to IFN-JAK/STAT1 pathway, IL6-JAK/STAT3 signalling controls also PML gene expression. Since IL6 is a well known signalling molecule that mobilizes organism-protective systems including innate and acquired immunity, our results indicate, that IL6 also can help to protect the organism by activation of PML tumour suppressor gene.

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

Senescent cells have many specific characteristics, but there is no universal marker characterizing all forms of senescence, which complicates detection of senescent cells especially in vivo, where senescence characteristic morphological changes or expression of B-galactosidase are hardly applicable. As discussed above, the increase in PML protein level was found in all type of senescent cells, but induction of PML transcription may also reflect not only senescence but other cellular responses to stress such as viral infection. Formation of senescence-associated heterochromatin foci (SAHF) is accompanied with reduction of transcription in the cell including transcription of the genes necessary for cell cycle progression[58]. SAHF were observed in several forms of cellular senescence, which makes them the candidate for the universal senescence marker. However, previous studies focused mostly on the mechanism of SAHF formation, such as involvement of pRb pathway in SAHF formation or mechanisms of chromatin modifications, the role of SAHF in transcriptional control of the cell cycle promoting genes, all mainly in the context of oncogene-induced senescence. Since SAHF have been studied mostly using fibroblasts, which are in fact not the best model for studying cancer pathologies that most frequently affect epithelial cells, we wanted to examine the relevance of SAHF in a broader spectrum of cell types, including neonatal human epidermal keratinocytes (HEKn), furthermore

the occurrence of SAHF in various types of senescence and whether these structures are applicable for detection of cellular senescence in vivo.

First, our experiments showed that SAHF formation differs not only between the types of cellular senescence, but also varies among cell types exposed to the same senescence-inducing stimulus. We showed that some cells (MRC5) are more sensitive to formation of these structures during oncogene-induced as well as replicative or drug-induced senescence in comparison with others (BJ and HEK293). Importantly, we showed the formation of SAHF after treatment with several chemotherapeutic drugs or bacterial toxin produced by facultative pathogenic strains of Gram-negative bacteria *Haemophilus ducreyi* (i.e. cytolethal distending toxin, CDT[59]) in MRC5 fibroblasts, but not in BJ fibroblasts or normal keratinocytes.

Secondly, Bartkova et al. observed the presence of heterochromatinization markers H3K9Me3 and HP1gamma in clinical specimens of premalignant human lesions[60]. Therefore we investigated whether typical DNA-DAPI defined SAHF could be detected in human tissues and tumours. Experiments with paraffin sections prepared from fibroblasts undergoing oncogene-induced senescence and stained by DAPI showed that routine tissue processing might reduce, but not completely prevent the ability to detect SAHF. Using exactly the same procedure of immunofluorescent staining on series of sections from tissues of human urinary bladder tumours (n=19), normal human bladder (n=5), colon (n=16), colorectal adenomas (grade III, n=38) and colon carcinomas (n=44), no clear evidence of DNA-DAPI-defined formation of SAHF was observed despite the positive expression pattern of H3K9Me3, HP1gamma and senescence-associated beta-galactosidase activity. Thus our results suggest that identification of senescent cells through detection of DAPI-defined SAHF may not be feasible.

Finally, correlation between the ability to form SAHF and the increased level of p16INK4a in various types of senescence was studied. p16 is an important CDK inhibitor whose involvement in cellular senescence was described and explained by its ability to induce hypophosphorylation of the Rb protein. High expression of p16 was identified as a marker of senescent cells in the skin of old people[61]. However, the induction of senescence was also observed in cells with low level of p16 due to activation of p53/p21 axis and possibly other mechanisms activating cell cycle checkpoints. We found the activation of p16 strongly correlated with the ability of cells to form SAHF at particular conditions, especially after RAS induction, in

contrast for example to BJ cells, where virtually undetectable levels of p16 after treatment with various drugs or CDT correspond with their inability to form SAHF. These findings support previous observation that p16 is not essential for every mode of cellular senescence, but is critical for heterochromatinization, engagement of Rb and irreversible cell cycle arrest.

## 6. Conclusions

In this work we wanted to contribute to understanding molecular mechanisms of cellular senescence, the important tumorigenesis barrier, with the aim to identify the role of cytokines and cytokine controlled signalling pathways in functional status of senescent cells.

This work brings new findings, which can be summarized as follows:

- commonly used chemotherapeutic drugs are able to induce premature cellular senescence in normal and cancer cells accompanied with the activation of complex cytokine network including many pro-inflammatory cytokines
- autocrine/paracrine signalling mediated by these cytokines helps to induce and maintain senescence phenotype including the expression of tumour suppressor genes in senescent cells
- activation of JAK/STAT signalling pathways in senescent cells contributes to maintenance of cytokine environment by regulation of expression of various cytokines, nonetheless does not play the crucial role in induction of drug-induced senescence
- secretion of cytokines by senescent cells and their autocrine/paracrine effects through JAK/STAT pathways play the essential role in activation of PML gene transcription rather than by induction via DNA damage response-activated p53
- IL6 is not essential for maintenance of drug-induced senescence, in contrary to oncogene-induced senescence
- apart from genotoxic stress-induced senescence phenotype, cytokine driven JAK/STAT3 activation plays the important role in induction of PML gene expression also under the unperturbed condition
- formation of senescence-associated heterochromatinization is not universal for all types of senescence and is not necessary for induction of senescence, namely for drug-induced and replicative senescence
- formation of SAHF correlates with induction of p16, which underscores their causal role in maintenance of senescence mediated through p16/Rb pathway



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## 8. Curriculum vitae

### Education:

- 09/2007 – 10/2012 Ph.D. degree in Molecular and Cellular Biology, Genetics and Virology at Charles University in Prague, Faculty of Natural Sciences, Czech Republic  
Thesis: DNA damage and signalling pathways in cellular senescence  
Supervisor: Zdeněk Hodný, M.D., Ph.D.
- 09/2002 – 06/2007 Master's degree in Biology at Charles University in Prague, Faculty of Natural Sciences, Czech Republic  
Diploma thesis: Comparison of minimal residual disease detection by flow cytometry and quantitative PCR in children with acute lymphoblastic leukemia  
Supervisor: Prof. Jan Trka M.D., Ph.D.

### Research Experience:

- 09/2007 – current Ph.D. student at the Department of Genome Integrity, Institute of Molecular Genetics, v.v.i., Academy of Sciences of the Czech Republic, Czech Republic
- 09/2004 – 06/2007 M.Sc. student at Childhood Leukaemia Investigation Prague, Department of Pediatric Hematology/Oncology, 2nd Faculty of Medicine, Charles University Prague, Czech Republic

### Activities related to Ph.D. study:

- 1) Inflammation and Cancer, Milan, November 7-9, 2007; poster presentation
- 2) XXIII. Olomoucké hematologické dny - 2nd Symposium on Advances in Molecular Hematology, Olomouc, 24. - 26. 6. 2009, oral presentation
- 3) 1st annual conference of genome stability and apoptosis, 2008, High Tatras, oral presentation
- 4) FEBS-Special Meeting 2010 - Jak-Stat Signalling: from Basics to Disease, poster presentation
- 5) Senescence in Cancer Meeting, 03 - 04 June 2011, Berlin, poster presentation
- 6) Central European Meeting on Genome Stability and Dynamics, 13. 5. 2011, Bratislava, oral presentation
- 7) New Concepts in Cancer Metastasis, Lisbon, Portugal, July 25 - 28, 2011, poster presentation

## 9. List of publications

**#1 Cytokine expression and signaling in drug-induced cellular senescence.**

Novakova Z, **Hubackova S**, Kosar M, Janderova-Rossmeslova L, Dobrovolna J, Vasicova P, Vancurova M, Horejsi Z, Hozak P, Bartek J, Hodny Z.

Oncogene. 2010 Jan 14;29(2):273-84. IF: 7.4

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

**Hubackova S**, Novakova Z, Krejcikova K, Kosar M, Dobrovolna J, Duskova P, Hanzlikova H, Vancurova M, Barath P, Bartek J, Hodny Z.

Cell Cycle. 2010 Aug 1;9(15):3085-99, IF: 4.99

**#3 Interleukin 6 signaling regulates PML gene expression in human normal and cancer cells.**

**Sona Hubackova**, Katerina Krejcikova, Jiri Bartek and Zdenek Hodny

J Biol Chem. 2012 Aug 3;287(32):26702-14. Epub 2012 Jun 18. IF: 5.3

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

Kosar M, Bartkova J, **Hubackova S**, Hodny Z, Lukas J, Bartek J.

Cell Cycle. 2011 Feb 1;10(3):457-68, IF: 4.99

**#5 Cytokines shape chemotherapy-induced and 'bystander' senescence.**

Hodny Z, **Hubackova S**, Bartek J.

Aging (Albany NY). 2010 Jul;2(7):375-6 review. IF: 5.127