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The function and regulation of senescent stromal-derived OPN

By

Ermira Pazolli

A dissertation presented to the
Graduate School of Arts and Sciences
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in partial fulfillment of the
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ABSTRACT OF THE DISSERTATION

The function and regulation of senescent stromal-derived osteopontin

By

Ermira Pazolli

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(Molecular Cell Biology)

Washington University in St. Louis, 2010

Dr. Sheila A. Stewart, Chairperson

Our understanding of tumors as complex organs has increased our appreciation for each component of the tumor microenvironment and its respective contribution to tumorigenesis. Fibroblasts found within a tumor actively participate in the growth, progression and metastasis of cancer cells. Senescent fibroblasts, which are permanently arrested yet metabolically active, accumulate in tissue over time where they may promote the proliferation and malignant conversion of preneoplastic cells in older individuals. I have examined the relationship between senescent fibroblasts and preneoplastic keratinocytes and identified the secreted multifunctional protein osteopontin (OPN) as a critical stromal mediating factor. RNAi-directed reduction of stromal OPN leads to decreased growth of preneoplastic keratinocytes *in vitro* and *in vivo.* Furthermore, I have demonstrated the presence of senescent stroma and associated OPN expression in the early stages of a chemical carcinogenesis mouse model, suggesting that it plays a role early in tumor formation. OPN is part of a unique transcriptional profile activated upon

senescence induction. Given the significance of senescent stromal-derived factors in tumorigenesis, I have investigated the regulation of OPN and other members of the senescence-associated secretory profile (SASP). I demonstrate that SASP is coordinately upregulated in response to multiple stimuli inducing senescence namely DNA damage, chromatin modulation and ectopic expression of p27, a cyclin-dependent kinase inhibitor. However, my work on the transcriptional activation of OPN reveals that SASP contains distinct subsets governed by unique transcriptional mechanisms. To this end, I demonstrate that OPN, unlike IL6 and IL8 – two well characterized SASP members – does not require the activity of ATM or NfκB for its expression in senescence. To uncover specific activators of OPN transcription, I examined OPN's promoter and I identified a fragment that is responsive in senescent fibroblasts. Furthermore, I demonstrate that OPN transcription requires chromatin modulators. Together my work presents a model where chromatin modifications in senescence play a central role in dictating transcriptional responses that ultimately impact the tumor microenvironment.

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Chapter 1: Background and Significance

1.1 Significance and Overview

Tumorigenesis is the result of cell autonomous alterations in the incipient tumor cell and alterations in the surrounding tissue. Elegant work *in vitro* has demonstrated that disruption of five essential pathways is sufficient to transform a normal cell [1]. Specifically, deregulation of tumor suppressor function, acquisition of constitutive mitogenic stimulus, disruption of protein phosphatase 2A function and acquisition of cellular immortality by stabilization and maintenance of telomeres is sufficient to transform normal human cells [1]. Despite the cell-autonomous changes driving transformation, research in the recent decade has demonstrated that a tumor operates as a complex organ consisting of multiple cell types with each contributing to tumor progression. Such intricate balance and interaction is required for malignant progression and metastasis.

1.2 The tumor microenvironment

The earliest descriptions of the tumor microenvironment involved components of the immune system. It was presumed that the immune system's main function in tumorigenesis was protective; however, some of its arms given the right molecular cues are potent accomplices in malignancy. Mounting evidence supports the pro-tumorigenic role of macrophages, which can be "trained" by the microenvironment to stimulate the growth of epithelial cells. To this end, presence of tumor associated macrophages (TAMs) has prognostic value with higher infiltration correlating with poor patient outcome [2]. Similar observations have been made for neutrophils found within a

malignant lesion [3]. Likewise, other elements of the immune system such as B cells, which constitute among the highest infiltrate in tumors, are critical for transformation and progression in well-characterized skin cancer models [4]. The complexity of the interactions in the tumor microenvironment is best illustrated by the elegant crosstalk between the adaptive and innate arm of the immune system. In one model, B cells found within the tumor release soluble immunoglobulin thereby mediating the recruitment of mast cells and pro-tumor macrophages [5]. Infiltration of innate immune cells induces enhanced neoplastic cell proliferation and angiogenesis and contributes to metastasis. Growing research on the tumor microenvironment has revealed an extensive array of paracrine players including adipocytes [6] and endothelial cells [7]. The microenvironment is composed of these cell types, which convert classically anti-tumor mechanisms into pro-tumor devices.

Identifying and elucidating the molecular actions of stromal-derived factors is valuable not only to functionally unravel molecular pathways involved in tumorigenesis, but also to identify molecular markers for diagnosis and therapy. In the past, tumor profiling consisted of collecting whole tumor tissue without separating tumor cells from the surrounding stroma thus generating a generic genetic landscape. However, with the advent of laser capture microdissection technology, it is possible to separate the tumor stroma from the epithelial cells. Surprisingly, this approach had recently yielded a genetic signature that is more reliable in predicting patient outcome than the classical signatures hitherto used [8]. Gene expression comparison of stroma found within the margin of invasive breast tumors to stroma distant to the tumor revealed a 26-gene

signature referred to as the stroma-derived prognostic predictor (SDPP). Application of SDPP to existing data sets collected from whole tumors accurately identified patients with a poor outcome. Furthermore, the stromal signature reflects different biological inputs including the immune system, fibroblastic response and extracellular matrix remodeling possibly facilitating the identification of patients that will benefit from certain therapies.

The impetus for generating stromal signatures was based on fundamental work performed on tumor microenvironmental constituents. A decade ago, fibroblasts isolated from a tumor (hereafter referred to "cancer-associated fibroblasts – CAFs") were shown to be potent promoters of tumorigenesis [9]. CAFs from prostate cancers, as opposed to fibroblasts isolated from normal prostate, stimulated the growth of initiated prostate epithelial cells – the latter were isolated from dysplastic prostate tissue showing no signs of carcinoma. CAFs altered the morphology of the initiated epithelial cells, protected them from death signals and increased their proliferation. In xenografts, CAFs contributed to enhanced tumors. Another seminal finding of the above study highlighted a critical aspect of the relationship between epithelial cells and activated stroma. When CAFs were incubated with normal prostate epithelial cells, although some morphological alterations occurred in the epithelial cells, there was no increased proliferation or tumor growth indicating that the response to activated stroma requires existing genetic mutations in the epithelium – an uninitiated epithelium is refractory to aberrant signaling emanating from the stromal compartment. Presently, it is unclear what mutations enable the epithelium to respond to paracrine stimulation.

While the origin of CAFs is still unknown (they could be derived from bone marrow progenitor cells or educated by the local milieu) and their genetic identity is controversial (opposing views contend whether CAFs have somatic mutations or result from epigenetic reprogramming) [10-12], their active role in tumorigenesis is undisputable. Following the Olumi et al study [9], numerous reports have demonstrated that CAFs have an altered expression profile enriched in growth factors, chemokines, extracellular matrix (ECM) remodeling enzymes and angiogenic factors. Indeed, this has lead to the argument that CAFs behave as myofibroblasts – an activated fibroblast that orchestrates tissue remodeling during wound healing [13].

The list of CAF-derived factors vested with tumor-altering properties is extensive: to this end, it was shown that stromal-derived factor 1 (SDF-1), which is overexpressed in CAFs acts upon epithelial cells inducing proliferation; moreover, it recruits from the bone marrow other components of the tumor microenvironment such as endothelial progenitor cells (EPCs) thus enhancing angiogenesis [14]. A variety of chemokines such as IL6, CCL5 and CXCL14 enlist elements of the immune system, which as described above play an active role in tumor progression. Mitogenic factors including EGF, HGF, PDGF and IGF promote the proliferation of cancer cells and enhance their survival. Abundant secretion of ECM remodeling enzymes such as matrix metalloproteinases (e.g. MMP2, 3, 9) and crosslinking enzymes (LOX) ensures the cooperation of the microenvironment for migration and invasion [15]. In fact, activated fibroblasts establish a migratory track for cancer cells thus mediating invasion and metastasis [16]. These and other molecular

processes not mentioned here underscore the fibroblasts as active participants in tumorigenesis.

The extensive profiling of cancer-associated fibroblasts and their role in tumorigenesis drew attention to another class of fibroblasts that had been studied for a long time in culture: senescent fibroblasts. Hayflick's seminal work demonstrated that primary cells such as fibroblasts have a finite lifespan (also referred to as the Hayflick limit) [17]. Later this "clocking" mechanism was identified as the telomere [18], which prevented the cell from undergoing an infinite number of divisions unless telomerase (the telomere extending enzyme) was present such as is the case with cancer cells. Furthermore, exposure to nonphysiological conditions such as abnormal oxygen levels, cellular stress or the presence of oncogenes translates into a permanent arrest indistinguishable from the Hayflick limit. All of these features had been well characterized in culture but it was not until recently that an array of elegant studies taking advantage of improved detection techniques and markers has demonstrated that senescent cells are indeed viable in tissue and perform important functions (to be discussed later).

One of the best accepted markers of senescence to date (although limited) is senescence-associated β-galactosidase. Originally described by Dimri et al [19] over a decade ago, it has become a universal indicator of senescence. In addition to being able to discriminate between quiescent or terminally differentiated cells and senescent cells, the authors reported an age-dependent increase in the number of senescent cells in samples of human skin suggesting senescent cells accumulate with age [19]. Although the number of senescent cells in tissue varies widely, the original findings have found

support in subsequent studies using additional markers of senescence such as telomere dysfunction or activation of the DNA damage response that clearly demonstrate an increase of senescent cells with age and in association with some pathologies [20, 21].

1.3 Senescence as a phenomenon of antagonistic pleitropy

While the link between senescence and organismal ageing is still debatable, the increased presence of senescent cells with age is well-established [21]. Additionally, the correlation between ageing and cancer onset is well established [22]. Indeed, the single largest risk factor for cancer is age. The molecular basis for increased cancer as a function of age remains incompletely understood. The cell autonomous mutation theory partially explains the increased cancer risk with the accumulation of mutations over time; however it has become clear that the surrounding environment is not a bystander in this process and can accelerate tumorigenesis. Pioneering work by Campisi and colleagues began to address the link between cancer and ageing by exploring the role of senescent fibroblasts given their accumulation in ageing and their "tumor-promoting" profile. Human primary fibroblasts driven to senescence either by replicative exhaustion, oncogene overexpression (Ras), induction of a tumor suppressor (p14 – ARF), or treatment with hydrogen peroxide were shown to enhance proliferation of epithelial cells in coculture systems *in vitro* and to stimulate tumor formation in xenografts [23]. This finding indicated that senescent in fibroblasts function as a potent paracrine tumor promoter. Similar to the original findings with CAFs, senescent fibroblasts only promoted the growth of premalignant and malignant epithelium but not normal epithelial

cells. Strikingly, paracrine stimulation occurred even when only ten percent of the fibroblast population was senescent and it employed both cell-cell contact and secreted factors [23].

The observation that senescence acts as a tumor promoter in a paracrine setting was groundbreaking because senescence was previously considered a tumor suppressor given it inhibits proliferation by permanently arresting cell proliferation. It has been argued that this is a classical example of evolutionary antagonistic pleiotrophy: senescence is beneficial early in life where it constitutes a potent anti-tumor mechanism within incipient tumor cells, but becomes detrimental in ageing due to its ability to alter the microenvironment and promote tumorigenesis [23]. The validity of senescence as a tumorigenesis barrier within the epithelial compartment has been confirmed by several studies. For example Eμ-N-Ras transgenic mice develop invasive lymphomas much earlier when they harbor deletions in the Suv39h1 (histone methyltransferase) or p53 locus and are unable to induce senescence. In contrast, Eμ-N-Ras mice with a wild type background display non-lymphoid neoplasias at a significant delay, which show signs of senescence [24] indicating that heterochromatin- and p53-mediated senescence stalls tumor formation.

Likewise, oncogene-induced senescence (OIS) initiated by $BRAF^{V600E}$ expression is found in human nevi, which display robust senescence markers and rarely progress to malignancy arguing that OIS is encountered physiologically and acts as a tumor barrier [25]. OIS was further shown to be a distinguishing characteristic of premalignant lesions (adenomas) and absent in malignant ones (adenocarcinomas). Meticulous profiling of the

progressing tumors delivered a histochemical signature of senescence thus allowing a more accurate diagnosis of senescence *in vivo* [26]. Finally, Pandolfi and colleagues demonstrated that the acute loss of PTEN results in senescence and late onset premalignant prostate lesions, whereas the combined loss of PTEN and p53 accelerates tumorigenesis and ensuing death by abrogating senescence. The rodent studies were supported by the identification of senescent cells in human prostate premalignant lesions [27]. These revolutionary studies heralded an era of active research in senescence as a physiological tumor-preventing mechanism [28, 29].

While on the surface, senescence may appear to have contradictory roles – both suppressive and promoting (**Figure 1.1**) – it is no different than other well-studied phenomena that are context dependent such as organismal immunity discussed earlier. The initial findings describing senescence as a putative promoting player in the tumor microenvironment were followed by closer examination of the expression profile of senescent fibroblast and their properties. Thus work from several groups has demonstrated that senescent fibroblasts secrete a plethora of growth factors such as amphiregulin, which is important for the proliferation of premalignant prostate epithelial cells [30]. Moreover, some growth factors originating from the tumor cells such as growth-regulated oncogene 1 (Gro-1) can induce senescence in neighboring fibroblasts generating a stroma conducive to further malignant progression [31]. Gro-1 is overexpressed by ovarian cancer cells and examination of human ovarian tumors revealed the presence of senescent stroma adjacent to the tumor suggesting that such cross-talk is present in human tumors.

Prominent inflammatory chemokines like IL6 and IL8 have become the hallmarks of what is now termed the senescence-associated secretory phenotype or SASP [32]. Not only do these chemokines act on neighboring epithelial cells by inducing proliferation and invasion and presumably on immune cells, but they are also components of a feedback mechanism reinforcing senescence [33]. Additionally, senescent stromal-derived MMP3 has been shown to promote branching of preneoplastic mammary epithelial cells and invasion in three-dimensional assays [34]. In another report, MMPs were responsible for the early growth advantage of xenografts injected with senescent fibroblasts [35]. In addition to mitogens and ECM remodeling enzymes, senescent fibroblasts also secrete proangiogenic factors such as vascular endothelial growth factor (VEGF), which may explain their ability to enhance endothelial cell invasion through a basement membrane [36]. Another outcome of paracrine signaling originating from senescent fibroblasts is increased radioresistance and drug resistance in the cancer cells underscoring senescent fibroblasts as potential therapeutic targets [37].

1.4 Osteopontin as a senescent stromal-derived factor

The above studies, demonstrating the ability of senescent fibroblasts to affect multiple growth parameters in the tumor microenvironment, provided the impetus to dissect the molecular mechanisms of cross-talk in the microenvironment. Examination of the expression profile of senescent fibroblasts in different systems has revealed some convergence. However, we performed independent microarray analysis in order to capture a transcriptional signature reflected by the culture conditions used in our system

(serum-free). This work led to the identification of osteopontin (OPN) [38], which is the focus of my thesis work. The following chapters will summarize my findings, but below I provide a basic introduction to osteopontin and its known functions in tissue homeostasis and its role in tumorigenesis.

OPN is a secreted protein with pleiotropic functions. Initially identified in the late 1970s as a protein associated with transformation [39], OPN is embedded abundantly in the bone matrix contributing to its calcification and ECM stability in addition to affecting osteoclast differentiation and bone resorption [40]. Later, its role in the immune system was characterized where it modifies inflammatory responses in a context-dependent fashion by its ability to act as a chemoattractant for macrophages, dendritic cells and T cells and enhances immunoglobulin production by B cells [41]. The outcome of OPN signaling in the immune system points to its role as a general "stress sensor" ultimately coordinating actions to control the damage response in tissue [40]. This model is supported by the unrestrained infections and inflammatory phenotype observed in the OPN null mouse [41], yet elevated levels of OPN have been reported in several pathologies associated with inflammation (e.g. Crohn's disease) affirming the complex and context-dependent role of OPN [42, 43].

The cytokine-like properties of OPN are most evident in tumorigenesis. OPN interacts with a multitude of receptors such as integrins ($\alpha_v\beta_1/\beta_3/\beta_5$ and $\alpha_4\beta_5/\beta_8$ and $\alpha_9\beta_1$) and particular variants of CD44 (v6 and v7). These interactions result in the activation of numerous signaling pathways leading to increased cell survival, proliferation, angiogenesis, cell motility and metastasis [44] hence, it is no surprise that OPN is

considered a prognostic marker for several malignancies [45]. In addition to multiple receptor utilization, signaling versatility is provided by the extensive array of modifications such as phosphorylation, cleavage by thrombin and several MMPs, and N- and O-linked glycosylation, which impact OPN function in a cell and tissue-dependent fashion. OPN's role in tumorigenesis is further supported by the lower incidence and delayed appearance of lesions in OPN-/- mice in a two-stage chemical carcinogenesis model [46]. Moreover, a recent finding identifies OPN secreted by cancer cells as a systemic pro-tumor effector. Actively growing tumors stimulate the proliferation of indolent tumor cells located in distant sites via the recruitment and local integration of bone marrow cells (BMCs). Systemic secretion of OPN by the growing tumors was responsible for the recruitment of BMCs and the subsequent proliferation of indolent tumor cells. Thus OPN impacts tumor growth not only locally but also systemically implicating it in micrometastases and recurrence [47].

Most of the studies conducted thus far have focused on epithelial-derived OPN highlighting its multifaceted roles in biology. However, it has already been mentioned that the stroma, represented mainly by immune cells [48], is a significant source of OPN in the microenvironment raising the possibility that fibroblast-derived OPN is an important contributor to tumorigenesis. This is based on several lines of evidence. Expression of platelet-derived growth factor (PDGF) by tumor cells induces recruitment of CAFs, which in turn augment tumor growth and angiogenesis via OPN [49]. Furthermore, OPN is a validated member of the stroma-derived prognostic predictor (SDPP) signature described earlier, thus solidifying its connection with stromal

involvement in tumorigenesis [8]. The potential role of senescent stromal-derived OPN as a paracrine effector of tumorigenesis will be addressed in Chapter 2 of this thesis.

1.5 Pathways and transcription factors involved in the senescence program

Given that senescent cells play a pivotal role in preneoplastic cell growth, it is critical to understand how senescent cells activate OPN and the rest of the senescent associated secretory phenotype (SASP). These molecules have a profound effect on the remodeling of the microenvironment, yet little is known as to their regulation in senescence. We and others have demonstrated that the secretory program is a transcriptional response [32, 33, 38, 50]. Thus far, our analysis suggests that these genes are coordinately regulated and that their expression is linked to the senescence program. Therefore, it follows that transcription factors required for the induction and maintenance of senescence may be responsible for the upregulation of the paracrine effectors as well. The p53 and Rb pathways are essential for senescence in human cells [51]. Upon major assaults such as critical telomere shortening, excessive DNA damage, aberrant mitogenic stimulation or other types of cellular stress, p53 is stabilized via different mechanisms (acetylation, phosphorylation, degradation of inhibitors [52]. Through its activation of p21 or through the direct control of other targets such as cyclins and associated kinases, p53 accomplishes cell cycle arrest and senescence.

In human cells, absence of p53 alone does not jeopardize entry into senescence due to the presence of signaling emanating from the p16-Rb pathway [53]. p16 is activated by a variety of stimuli – it inhibits cyclin-dependent kinases 4 and 6, therefore

blocking the phosphorylation of Rb . Hypophosphorylated Rb interacts with E2F repressing proliferation [54]. In several cell types (but not all) senescence is accompanied by distinct heterochromatic domains known as senescence-associated heterochromatic foci (SAHFs), which occupy E2F-controlled promoters and are dependent on p16/Rb [55, 56]. In addition to Rb, other proteins contribute to heterochromatin present in SAHFs. Histone chaperones HIRA and ASF-1 translocate to PML bodies upon a senescence trigger and this localization is a preliminary step required for SAHFs formation [57, 58]. SAHFs contain other nonhistone protein classes: HP1, which is enriched in heterochromatic regions containing H3K9-me3 contributing to chromatin assembly and epigenetic modifications; and unexpectedly HMGA proteins usually associated with proliferation [55, 56]. Such facultative heterochromatinization enforces a permanent cellular arrest and may explain the classic insensitivity to mitogenic stimuli observed in senescent cells. It is yet unclear as to whether SAHFs are a cause or consequence of senescence, although work mentioned previously demonstrates that histone methyltransferases are required for senescence induction *in vivo* [24].

Such chromatin changes in senescence are not surprising given the long-known nuclear architecture and chromatin aberrations observed in cells isolated from patients with premature ageing syndromes or aged model organisms consisting of loss of heterochromatic structure, reduction of HP1, global decrease in methylation, decrease in deacetylase activity, redistribution of silencing complexes and persistent DNA damage ([59] and references therein) accompanied by significant changes in gene expression patterns with an overrepresentation of stress response genes [60]. While the trigger for

the above changes is not known, these phenomena are interlinked as chromatin relaxation activates components of the DNA damage response (DDR) [61] and DNA breaks alter surrounding chromatin [62] – both processes impact transcription [59]. A recent surprising finding is that DDR activates a transcriptional response not directly associated with the repair of the breaks themselves in developing lymphocytes [63] raising the intriguing possibility that a similar phenomenon occurs in senescence. In fact, both replicative senescence (due to dysfunctional telomeres) [64] and OIS (due to hyperreplication) [65, 66] are characterized by robust DDR activation, which has been implicated in the maintenance of the senescent state. Recent work has suggested that such signaling is also important for the activation of a subset of SASP factors [67]. Currently, it is unclear whether OPN is under the same regulatory mechanisms described above; therefore, I undertook to investigate the interplay between DDR and chromatin and their putative role in the transcriptional regulation of OPN and other SASP components. This work will be addressed in Chapter 3.

In addition to p53 and Rb, other transcription factors have been invoked in the establishment of senescence and the secretory phenotype associated with it (**Figure 1.3**). C/EBPβ, a bZIP transcription factor, translates extracellular cues into proliferative, tumorigenic, arresting, differentiation and metabolic responses. It is activated downstream of physiologic Ras signaling [68]. In a similar fashion, it is activated by and required for the execution of oncogene-induced senescence (OIS) in mouse embryonic fibroblasts [69]. Recent reports demonstrate that this is the case in human cells as well. OIS ($BRAF^{V600E}$ or Mek-driven) in human fibroblasts upregulates the levels of C/EBPβ,

which enforces cellular arrest via its transcriptional control of cytokines such as IL6 and IL8 [33, 50]. As already noted, senescent cells have a pronounced inflammatory profile and one of the main transcription factors involved in cytokine production and inflammation is NfκB [70], which cooperates in the activation of SASP [50, 71]. Thus, the inflammatory transcriptome partially controlled in senescence by C/EBPβ and NfκB fulfills a dual mission: 1) maintains the permanent arrest by a self-amplifying secretory loop; and 2) modifies the surrounding microenvironment thereby promoting tumorigenesis.

The transcriptional regulation of OPN is complex and context-dependent reflecting its role in diverse physiological processes. A cursory examination of the human OPN promoter reveals binding sites for multiple transcription factors [72], a few of which occupy these sites *in vivo*. Based on previous work analyzing the OPN promoter and the abovementioned literature about transcriptional control in senescence, I postulated that some of these mechanisms governed OPN transcription; however, I also decided to undertake a nonbiased, classical promoter analysis approach: I investigated OPN promoter activity in senescent cells, identifying the responsive fragment and transcription factor binding sites contained therein. Information derived from this study and the subsequent experiments are contained in Chapter 3 and Appendix 1 and 2. This work reveals layers of regulation in OPN and SASP transcription, highlighting points of convergence but also uniqueness.

1.6 Summary

The more we delve into a physiological process, the more we realize biology is multi-faceted and highly plastic. We are yet at the beginning of understanding the molecular mechanisms of senescence induction and enforcement and its biological implications but significant strides have been made in this direction. Senescent fibroblasts have been shown to promote preneoplastic epithelial growth and formation of tumors *in vivo*, while their genetically identical young counterparts cannot. Even though senescent fibroblasts are genetically normal, they appear to play a role in tumorigenesis in a paracrine fashion. These observations indicate that senescent fibroblasts may be a critical component of the tumor microenvironment. Additionally, they may provide an experimental basis that links ageing and cancer. Conversely, senescent cells have been shown to accumulate with age as do preneoplastic cells [21] (cells that carry mutations that are not on their own sufficient to transform the cell); therefore, the two may come into proximity of each other increasing the opportunity for a crosstalk between senescent stroma and initiated epithelium. Such interactions may contribute to progression to full tumorigenesis. If we are to understand how the stromal compartment contributes to the transformation process we must identify factors that modulate the growth of preneoplastic cells. This work will have a substantial impact in our understanding of carcinogenesis, particularly in aged individuals. In addition, because this work focuses on the genetically normal stromal compartment it may identify novel anti-neoplastic targets that are less likely to be lost to genetic mutation; a problem often encountered when targeting the cancer cell itself.

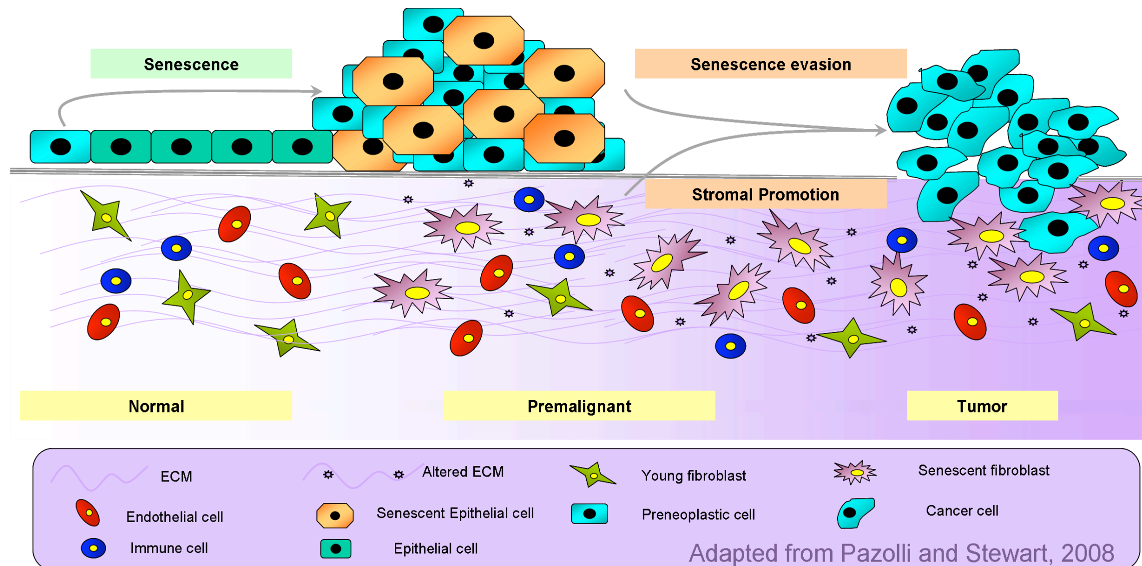

Figure 1.1 Dual role of senescence in tumorigenesis
Upon telomere erosion or cellular stress, cells engage the senescence response, which results in a proliferative arrest and an altered expression profile. In a tissue setting, an oncogenic assault triggers checkpoint activation and senescence. Such genetic assaults accumulate over time leading to an increase in preneoplastic cells. Those preneoplastic cells that evade the senescence checkpoints are now primed to progress to malignancy. Similarly, senescence is triggered in the stroma as a function of acute and cumulative stress. In fact senescent fibroblasts alter the surrounding microenvironment by secreting matrix remodeling proteins and growth factors thus contributing to the transformation process. Evasion of senescence by the would-be tumor cell in combination with a promoting stroma leads to tumorigenesis.

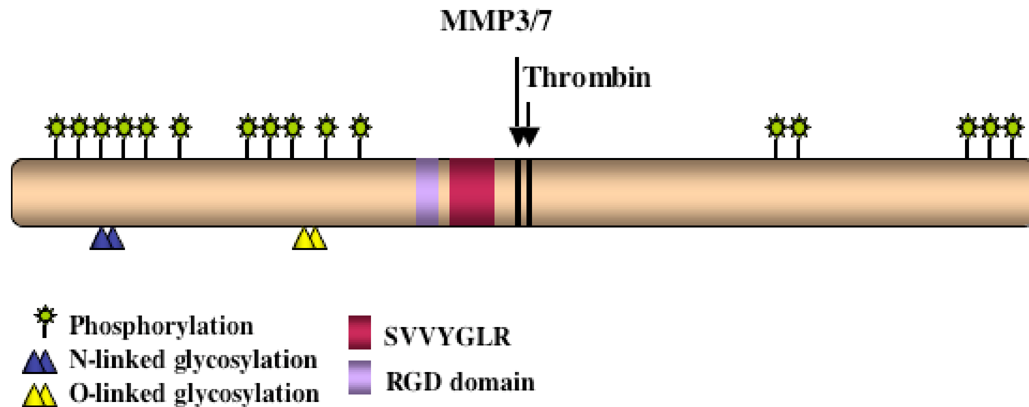

Figure 1.2 Structure of the human osteopontin protein. Osteopontin contains multiple phosphorylation and glycosylation sites. Additionally, OPN is subject to cleavage by thrombin, which reveals a cryptic "SVVYGLR" site that is recognized by $\alpha_4\beta_1$ and $\alpha_9\beta_1$. Matrix metalloproteases, MMP3 and 7 also cleave OPN, yielding several smaller fragments reported to have differential activity. Each of these modifications impact OPN activity in a tissue and context-dependent manner thus mediating a vast array of biological outcomes [41].

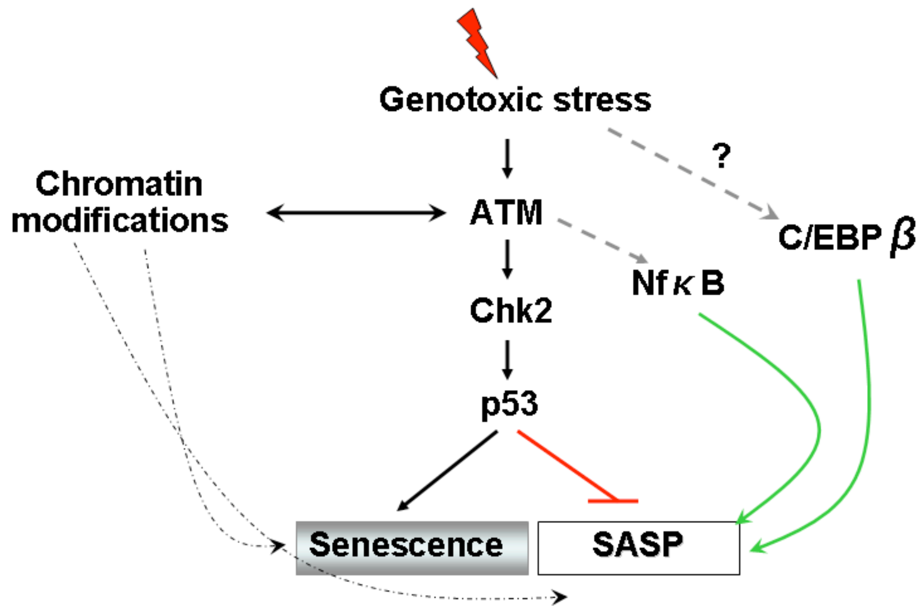

Figure 1.3 Regulatory mechanisms in senescence
Activation of the DNA damage response (DDR) upon DNA breaks or chromatin modifications results in induction of senescence mediated by p53. Although, p53 is required to establish senescence, it stifles the senescence-associated secretory response (SASP) [32]; whereas, NfκB and C/EBPβ directly transcribe SASP promoters [50]. It is currently unknown how these transcription factors are activated in senescence

References

1. Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999). Creation of human tumor cells with defined genetic elements. Nature *400*, 464-468.
2. Qian, B.Z., and Pollard, J.W. Macrophage diversity enhances tumor progression and metastasis. Cell *141*, 39-51.
3. Fridlender, Z.G., Sun, J., Kim, S., Kapoor, V., Cheng, G., Ling, L., Worthen, G.S., and Albelda, S.M. (2009). Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell *16*, 183-194.
4. de Visser, K.E., Korets, L.V., and Coussens, L.M. (2005). De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. Cancer Cell *7*, 411-423.
5. Andreu, P., Johansson, M., Affara, N.I., Pucci, F., Tan, T., Junankar, S., Korets, L., Lam, J., Tawfik, D., DeNardo, D.G., Naldini, L., de Visser, K.E., De Palma, M., and Coussens, L.M. FcRgamma activation regulates inflammation-associated squamous carcinogenesis. Cancer Cell *17*, 121-134.
6. Onuma, M., Bub, J.D., Rummel, T.L., and Iwamoto, Y. (2003). Prostate cancer cell-adipocyte interaction: leptin mediates androgen-independent prostate cancer cell proliferation through c-Jun NH2-terminal kinase. J Biol Chem *278*, 42660-42667.
7. Bertolini, F., Shaked, Y., Mancuso, P., and Kerbel, R.S. (2006). The multifaceted circulating endothelial cell in cancer: towards marker and target identification. Nat Rev Cancer *6*, 835-845.
8. Finak, G., Bertos, N., Pepin, F., Sadekova, S., Souleimanova, M., Zhao, H., Chen, H., Omeroglu, G., Meterissian, S., Omeroglu, A., Hallett, M., and Park, M. (2008). Stromal gene expression predicts clinical outcome in breast cancer. Nat Med *14*, 518-527.
9. Olumi, A.F., Grossfeld, G.D., Hayward, S.W., Carroll, P.R., Tlsty, T.D., and Cunha, G.R. (1999). Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res *59*, 5002-5011.
10. Haviv, I., Polyak, K., Qiu, W., Hu, M., and Campbell, I. (2009). Origin of carcinoma associated fibroblasts. Cell Cycle *8*, 589-595.
11. Qiu, W., Hu, M., Sridhar, A., Opeskin, K., Fox, S., Shipitsin, M., Trivett, M., Thompson, E.R., Ramakrishna, M., Gorringe, K.L., Polyak, K., Haviv, I., and Campbell, I.G. (2008). No evidence of clonal somatic genetic alterations in cancer-associated fibroblasts from human breast and ovarian carcinomas. Nat Genet *40*, 650-655.

12. Kurose, K., Gilley, K., Matsumoto, S., Watson, P.H., Zhou, X.P., and Eng, C. (2002). Frequent somatic mutations in PTEN and TP53 are mutually exclusive in the stroma of breast carcinomas. Nat Genet *32*, 355-357.
13. Kalluri, R., and Zeisberg, M. (2006). Fibroblasts in cancer. Nat Rev Cancer *6*, 392-401.
14. Orimo, A., Gupta, P.B., Sgroi, D.C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., Carey, V.J., Richardson, A.L., and Weinberg, R.A. (2005). Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell *121*, 335-348.
15. Ostman, A., and Augsten, M. (2009). Cancer-associated fibroblasts and tumor growth--bystanders turning into key players. Curr Opin Genet Dev *19*, 67-73.
16. Gaggioli, C., Hooper, S., Hidalgo-Carcedo, C., Grosse, R., Marshall, J.F., Harrington, K., and Sahai, E. (2007). Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat Cell Biol *9*, 1392-1400.
17. Hayflick, L. (1965). The Limited in Vitro Lifetime of Human Diploid Cell Strains. Exp Cell Res *37*, 614-636.
18. Harley, C.B., Futcher, A.B., and Greider, C.W. (1990). Telomeres shorten during ageing of human fibroblasts. Nature *345*, 458-460.
19. Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., and et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A *92*, 9363-9367.
20. Herbig, U., Ferreira, M., Condel, L., Carey, D., and Sedivy, J.M. (2006). Cellular senescence in aging primates. Science *311*, 1257.
21. Campisi, J., and d'Adda di Fagagna, F. (2007). Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol *8*, 729-740.
22. DePinho, R.A. (2000). The age of cancer. Nature *408*, 248-254.
23. Krtolica, A., Parrinello, S., Lockett, S., Desprez, P.Y., and Campisi, J. (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci U S A *98*, 12072-12077.
24. Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A.H., Schlegelberger, B., Stein, H., Dorken, B., Jenuwein, T., and Schmitt, C.A. (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. Nature *436*, 660-665.
25. Michaloglou, C., Vredeveld, L.C., Soengas, M.S., Denoyelle, C., Kuilman, T., van der Horst, C.M., Majoor, D.M., Shay, J.W., Mooi, W.J., and Peeper, D.S.

(2005). BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature *436*, 720-724.

26. Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A.J., Barradas, M., Benguria, A., Zaballos, A., Flores, J.M., Barbacid, M., Beach, D., and Serrano, M. (2005). Tumour biology: senescence in premalignant tumours. Nature *436*, 642.
27. Chen, Z., Trotman, L.C., Shaffer, D., Lin, H.K., Dotan, Z.A., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W., Cordon-Cardo, C., and Pandolfi, P.P. (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature *436*, 725-730.
28. Krizhanovsky, V., Yon, M., Dickins, R.A., Hearn, S., Simon, J., Miething, C., Yee, H., Zender, L., and Lowe, S.W. (2008). Senescence of activated stellate cells limits liver fibrosis. Cell *134*, 657-667.
29. Feldser, D.M., and Greider, C.W. (2007). Short telomeres limit tumor progression in vivo by inducing senescence. Cancer Cell *11*, 461-469.
30. Bavik, C., Coleman, I., Dean, J.P., Knudsen, B., Plymate, S., and Nelson, P.S. (2006). The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. Cancer Res *66*, 794-802.
31. Yang, G., Rosen, D.G., Zhang, Z., Bast, R.C., Jr., Mills, G.B., Colacino, J.A., Mercado-Uribe, I., and Liu, J. (2006). The chemokine growth-regulated oncogene 1 (Gro-1) links RAS signaling to the senescence of stromal fibroblasts and ovarian tumorigenesis. Proc Natl Acad Sci U S A *103*, 16472-16477.
32. Coppe, J.P., Patil, C.K., Rodier, F., Sun, Y., Munoz, D.P., Goldstein, J., Nelson, P.S., Desprez, P.Y., and Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol *6*, 2853-2868.
33. Kuilman, T., Michaloglou, C., Vredeveld, L.C., Douma, S., van Doorn, R., Desmet, C.J., Aarden, L.A., Mooi, W.J., and Peeper, D.S. (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell *133*, 1019-1031.
34. Parrinello, S., Coppe, J.P., Krtolica, A., and Campisi, J. (2005). Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. J Cell Sci *118*, 485-496.
35. Liu, D., and Hornsby, P.J. (2007). Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. Cancer Res *67*, 3117-3126.
36. Coppe, J.P., Kauser, K., Campisi, J., and Beausejour, C.M. (2006). Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. J Biol Chem *281*, 29568-29574.

37. Tsai, K.K., Stuart, J., Chuang, Y.Y., Little, J.B., and Yuan, Z.M. (2009). Low-dose radiation-induced senescent stromal fibroblasts render nearby breast cancer cells radioresistant. Radiat Res *172*, 306-313.

38. Pazolli, E., Luo, X., Brehm, S., Carbery, K., Chung, J.J., Prior, J.L., Doherty, J., Demehri, S., Salavaggione, L., Piwnica-Worms, D., and Stewart, S.A. (2009). Senescent stromal-derived osteopontin promotes preneoplastic cell growth. Cancer Res *69*, 1230-1239.

39. Senger, D.R., Wirth, D.F., and Hynes, R.O. (1979). Transformed mammalian cells secrete specific proteins and phosphoproteins. Cell *16*, 885-893.

40. Bulfone-Paus, S., and Paus, R. (2008). Osteopontin as a new player in mast cell biology. Eur J Immunol *38*, 338-341.

41. Denhardt, D.T., Noda, M., O'Regan, A.W., Pavlin, D., and Berman, J.S. (2001). Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. J Clin Invest *107*, 1055-1061.

42. Agnholt, J., Kelsen, J., Schack, L., Hvas, C.L., Dahlerup, J.F., and Sorensen, E.S. (2007). Osteopontin, a protein with cytokine-like properties, is associated with inflammation in Crohn's disease. Scand J Immunol *65*, 453-460.

43. Mishima, R., Takeshima, F., Sawai, T., Ohba, K., Ohnita, K., Isomoto, H., Omagari, K., Mizuta, Y., Ozono, Y., and Kohno, S. (2007). High plasma osteopontin levels in patients with inflammatory bowel disease. J Clin Gastroenterol *41*, 167-172.

44. Rangaswami, H., Bulbule, A., and Kundu, G.C. (2006). Osteopontin: role in cell signaling and cancer progression. Trends Cell Biol *16*, 79-87.

45. Johnston, N.I., Gunasekharan, V.K., Ravindranath, A., O'Connell, C., Johnston, P.G., and El-Tanani, M.K. (2008). Osteopontin as a target for cancer therapy. Front Biosci *13*, 4361-4372.

46. Hsieh, Y.H., Juliana, M.M., Hicks, P.H., Feng, G., Elmets, C., Liaw, L., and Chang, P.L. (2006). Papilloma development is delayed in osteopontin-null mice: implicating an antiapoptosis role for osteopontin. Cancer Res *66*, 7119-7127.

47. McAllister, S.S., Gifford, A.M., Greiner, A.L., Kelleher, S.P., Saelzler, M.P., Ince, T.A., Reinhardt, F., Harris, L.N., Hylander, B.L., Repasky, E.A., and Weinberg, R.A. (2008). Systemic endocrine instigation of indolent tumor growth requires osteopontin. Cell *133*, 994-1005.

48. Morimoto, J., Kon, S., Matsui, Y., and Uede, T. Osteopontin; as a target molecule for the treatment of inflammatory diseases. Curr Drug Targets *11*, 494-505.

49. Anderberg, C., Li, H., Fredriksson, L., Andrae, J., Betsholtz, C., Li, X., Eriksson, U., and Pietras, K. (2009). Paracrine signaling by platelet-derived growth factor-

CC promotes tumor growth by recruitment of cancer-associated fibroblasts. Cancer Res *69*, 369-378.

50. Acosta, J.C., O'Loghlen, A., Banito, A., Guijarro, M.V., Augert, A., Raguz, S., Fumagalli, M., Da Costa, M., Brown, C., Popov, N., Takatsu, Y., Melamed, J., d'Adda di Fagagna, F., Bernard, D., Hernando, E., and Gil, J. (2008). Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell *133*, 1006-1018.

51. Shay, J.W., Pereira-Smith, O.M., and Wright, W.E. (1991). A role for both RB and p53 in the regulation of human cellular senescence. Exp Cell Res *196*, 33-39.

52. Zuckerman, V., Wolyniec, K., Sionov, R.V., Haupt, S., and Haupt, Y. (2009). Tumour suppression by p53: the importance of apoptosis and cellular senescence. J Pathol *219*, 3-15.

53. Sakamoto, K., Howard, T., Ogryzko, V., Xu, N.Z., Corsico, C.C., Jones, D.H., and Howard, B. (1993). Relative mitogenic activities of wild-type and retinoblastoma binding-defective SV40 T antigens in serum-deprived and senescent human diploid fibroblasts. Oncogene *8*, 1887-1893.

54. Sherr, C.J., and McCormick, F. (2002). The RB and p53 pathways in cancer. Cancer Cell *2*, 103-112.

55. Narita, M., Nunez, S., Heard, E., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., and Lowe, S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell *113*, 703-716.

56. Narita, M., Krizhanovsky, V., Nunez, S., Chicas, A., Hearn, S.A., Myers, M.P., and Lowe, S.W. (2006). A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. Cell *126*, 503-514.

57. Ye, X., Zerlanko, B., Zhang, R., Somaiah, N., Lipinski, M., Salomoni, P., and Adams, P.D. (2007). Definition of pRB- and p53-dependent and -independent steps in HIRA/ASF1a-mediated formation of senescence-associated heterochromatin foci. Mol Cell Biol *27*, 2452-2465.

58. Zhang, R., Poustovoitov, M.V., Ye, X., Santos, H.A., Chen, W., Daganzo, S.M., Erzberger, J.P., Serebriiskii, I.G., Canutescu, A.A., Dunbrack, R.L., Pehrson, J.R., Berger, J.M., Kaufman, P.D., and Adams, P.D. (2005). Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Dev Cell *8*, 19-30.

59. Oberdoerffer, P., and Sinclair, D.A. (2007). The role of nuclear architecture in genomic instability and ageing. Nat Rev Mol Cell Biol *8*, 692-702.

60. Csoka, A.B., English, S.B., Simkevich, C.P., Ginzinger, D.G., Butte, A.J., Schatten, G.P., Rothman, F.G., and Sedivy, J.M. (2004). Genome-scale expression profiling of Hutchinson-Gilford progeria syndrome reveals widespread transcriptional misregulation leading to mesodermal/mesenchymal defects and accelerated atherosclerosis. Aging Cell *3*, 235-243.

61. Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature *421*, 499-506.

62. Iijima, K., Ohara, M., Seki, R., and Tauchi, H. (2008). Dancing on damaged chromatin: functions of ATM and the RAD50/MRE11/NBS1 complex in cellular responses to DNA damage. J Radiat Res (Tokyo) *49*, 451-464.

63. Bredemeyer, A.L., Helmink, B.A., Innes, C.L., Calderon, B., McGinnis, L.M., Mahowald, G.K., Gapud, E.J., Walker, L.M., Collins, J.B., Weaver, B.K., Mandik-Nayak, L., Schreiber, R.D., Allen, P.M., May, M.J., Paules, R.S., Bassing, C.H., and Sleckman, B.P. (2008). DNA double-strand breaks activate a multi-functional genetic program in developing lymphocytes. Nature *456*, 819-823.

64. von Zglinicki, T., Saretzki, G., Ladhoff, J., d'Adda di Fagagna, F., and Jackson, S.P. (2005). Human cell senescence as a DNA damage response. Mech Ageing Dev *126*, 111-117.

65. Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L.V., Kolettas, E., Niforou, K., Zoumpourlis, V.C., Takaoka, M., Nakagawa, H., Tort, F., Fugger, K., Johansson, F., Sehested, M., Andersen, C.L., Dyrskjot, L., Orntoft, T., Lukas, J., Kittas, C., Helleday, T., Halazonetis, T.D., Bartek, J., and Gorgoulis, V.G. (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature *444*, 633-637.

66. Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P.G., Bensimon, A., Maestro, R., Pelicci, P.G., and d'Adda di Fagagna, F. (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638-642.

67. Rodier, F., Coppe, J.P., Patil, C.K., Hoeijmakers, W.A., Munoz, D.P., Raza, S.R., Freund, A., Campeau, E., Davalos, A.R., and Campisi, J. (2009). Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. Nat Cell Biol *11*, 973-979.

68. Mo, X., Kowenz-Leutz, E., Xu, H., and Leutz, A. (2004). Ras induces mediator complex exchange on C/EBP beta. Mol Cell *13*, 241-250.

69. Sebastian, T., Malik, R., Thomas, S., Sage, J., and Johnson, P.F. (2005). C/EBPbeta cooperates with RB:E2F to implement Ras(V12)-induced cellular senescence. Embo J *24*, 3301-3312.

70. Pasparakis, M. (2009). Regulation of tissue homeostasis by NF-kappaB signalling: implications for inflammatory diseases. Nat Rev Immunol *9*, 778-788.

71. Orjalo, A.V., Bhaumik, D., Gengler, B.K., Scott, G.K., and Campisi, J. (2009). Cell surface-bound IL-1alpha is an upstream regulator of the senescence-

associated IL-6/IL-8 cytokine network. Proc Natl Acad Sci U S A *106*, 17031-17036.

72. Hijiya, N., Setoguchi, M., Matsuura, K., Higuchi, Y., Akizuki, S., and Yamamoto, S. (1994). Cloning and characterization of the human osteopontin gene and its promoter. Biochem J *303 (Pt 1)*, 255-262.

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Chapter 2: Senescent Stromal-Derived Osteopontin Promotes Preneoplastic Cell Growth

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E.Pazolli was the principal contributor to this work.

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Abstract

Alterations in the tissue microenvironment collaborate with cell autonomous genetic changes to contribute to neoplastic progression. The importance of the microenvironment in neoplastic progression is underscored by studies demonstrating that fibroblasts isolated from a tumor stimulate the growth of preneoplastic and neoplastic cells in xenograft models. Similarly, senescent fibroblasts promote preneoplastic cell growth *in vitro* and *in vivo*. Because senescent cells accumulate with age, their presence is hypothesized to facilitate preneoplastic cell growth and tumor formation in older individuals. To identify senescent stromal factors directly responsible for stimulating preneoplastic cell growth, we carried out whole genome transcriptional profiling and compared senescent fibroblasts to their younger counterparts. We identified osteopontin (OPN) as one of the most highly elevated transcripts in senescent fibroblasts. Importantly, reduction of OPN protein levels by RNAi did not impact senescence induction in fibroblasts; however, it dramatically reduced the growth-promoting activities of senescent fibroblasts *in vitro* and *in vivo*, demonstrating that OPN is necessary for paracrine stimulation of preneoplastic cell growth. In addition, we found that recombinant OPN was sufficient to stimulate preneoplastic cell growth. Finally, we demonstrate that OPN is expressed in senescent stroma within preneoplastic lesions that arise following DMBA/TPA treatment of mice, suggesting that stromal-derived OPN-mediated signaling events impact neoplastic progression.

Introduction

Age is the greatest risk factor for the development of neoplasia [1, 2]. The molecular and physiologic factors that underlie age-related increases in cancer incidence are diverse and include cell autonomous genetic and epigenetic alterations and coincident alterations in the tissue microenvironment. Genetic analysis of human cancers has revealed a myriad of mutations that disrupt diverse cellular signaling pathways. Cell culture systems and murine models have demonstrated that disruption of pathways that regulate tissue homeostasis, cell growth, cell survival/death and invasiveness cooperate to produce a transformed cell [3-5]. While these cell autonomous alterations are critical to tumorigenesis, preneoplastic and neoplastic lesions arise within the context of a complex stromal compartment that consists of numerous cell types. Among these are epithelial cells, fibroblasts, immune cells, and cells that contribute to angiogenesis that together work to enable transformation.

The importance of the host microenvironment in the tumorigenic process is supported by numerous studies. For example, it has been shown that chickens infected with the Rous sarcoma virus develop tumors at sites of injury [6] and that activation of mast cells enhances transformation in a skin model of carcinogenesis [7]. These studies suggest that the inflammatory response plays a key role in tumorigenesis. In further support of this hypothesis, other studies have demonstrated that the chronic inflammation caused by H. pylori infection or Crohn's disease predisposes affected individuals to cancer [8, 9]. In addition to inflammatory cells, fibroblasts can also promote tumorigenesis. Under normal conditions, fibroblasts are responsible for matrix

deposition and reorganization and facilitate recruitment of cells that participate in tissue homeostasis. However, fibroblasts present within neoplastic lesions display altered expression profiles compared to fibroblasts isolated from normal tissues. Indeed, the gene expression profiles found in cancer associated fibroblasts (CAFs) are reminiscent of those found within a wound, and include expression of numerous growth factors, chemokines, and angiogenic factors (reviewed in [10, 11]). A role for CAFs in promoting transformation was demonstrated in one study in which CAFs isolated from prostate carcinomas were found to stimulate the *in vitro* and *in vivo* growth of immortalized prostate epithelial cells whereas prostate fibroblasts distal to tumors had no such capacity [12].

In addition to the increased mutational load and presence of preneoplastic cells found to arise in aging epithelia, [13, 14], the stromal compartment also undergoes age-related changes. Indeed, senescent cells accumulate in tissues with age [15-17]. Analysis of senescent fibroblasts reveals a significantly altered gene expression profile relative to their younger counterparts [18, 19]. Interestingly this altered gene expression profile mirrors that observed during wound repair (reviewed in [10, 11]). Prominent among genes whose expression is dramatically altered during wound repair are those that remodel the extracellular matrix as well as growth factors and inflammatory cytokines. Moreover, senescent fibroblasts function analogously to CAFs in that they promote the *in vitro* and *in vivo* growth of preneoplastic cells [19, 20]. This latter observation suggests that the age-related accumulation of senescent stromal cells and the alterations that they produce within the tissue, collaborate with increasing numbers of preneoplastic cells to

influence cancer incidence in older individuals. However, how senescent stromal cells function to promote tumorigenesis is still poorly understood because the panoply of factors secreted by these cells has yet to be fully identified.

Our goal was to identify senescent stromal factors that impact preneoplastic cell growth in order to begin to delineate the molecular mechanisms by which senescent stroma functions in tumorigenesis. To this end, we performed expression profiling of replicative senescent (RS [21]) fibroblasts and found that numerous factors were coordinately modulated. This study identifies one of these factors, osteopontin (OPN) as a critical mediator of stromal-epithelial interactions both *in vitro* and *in vivo*. We demonstrate that OPN is sufficient to stimulate the growth of preneoplastic cells. Finally, we show that senescent stroma expressing OPN can be found within premalignant lesions *in vivo*, supporting the physiologic relevance of these results and the potential role of OPN in promoting tumorigenesis.

Materials and Methods

Cell Lines. Human foreskin BJ fibroblasts and 293T cells were grown as previously described [22]. HaCaT cells are spontaneously immortalized human keratinocytes; are aneuploid, nontumorigenic and retain a near normal pattern of differentiation [23]. They were grown in DME plus 10% heat-inactivated FCS. N.p.c.T human keratinocytes were a kind gift from James Rheinwald (Harvard, MA) and were grown as previously described [24]. N.p.c.T. are human epidermal keratinocytes that were derived from newborn foreskin and transduced with human telomerase, mutant CDK4 (R24C) [24].

These cells are immortal but retain the ability to undergo an epidermis-type suprabasal differentiation program as evidenced by the ability to form a granular cell layer and an enucleated stratum corneum [24].

Virus production and retroviral infections. Virus preparation as described [22].

Microarray analysis. BJ fibroblasts were mock or Bleomycin sulfate-treated (100ug/ml, Sigma, St. Louis, MO) for 24 hrs. Replicatively senescent fibroblasts were obtained by continuous passage. After 72 hr serum-starvation, RNA was collected using TRIzol (Invitrogen, Carlsbad, CA). Biotinylated cRNA was hybridized to Affymetrix Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA) by the Washington University Microarray Facility. There were 4, 6, and 6 samples for SIPS, RS, and young respectively. Microarray quality control, analysis, and clustering (UPGMA by Centroid) were performed using dChip (May, 2008 release) [25]. All GeneChip comparisons had a cut-off basis of a lower bound of 90% confidence of fold-change ≥1.5 and expression intensity difference ≥75. Gene Ontological categorization of probe sets was performed using the Gene Ontology database, current to publication date. GO terms used: Immune & Inflammation (IDs: 6955, 9615, 42742, 6952, 6954), Mitogens and Regulation of Proliferation (IDs: 7067, 85, 45840, 86, 82, 45930, 7049, 45786, 187, 186, 1558, 8283), Extracellular Matrix and Secreted Factors (IDs: 7596, 7160, 7155, 5604, 8243, 42730, 7596, 5201, 5581, 4252, 8237, 5125, 8008).

Co-Culture conditions. $1.2x10^4$ fibroblasts were plated in 96 well plates (Fisher Scientific, Pittsburgh, PA) and treated as described above. HaCaT- ($1.0\ x10^3$) or N.p.c.T- ($2.0x10^3$) CBR cells were plated on fibroblasts and incubated for 96 hr. Luciferase (Chroma-Glo Luciferase System, Promega) readings were performed in a Bio Tek microplate reader (Bio Tek Instruments, Winooski, VT).

OPN shRNA and Real-Time PCR. Three shRNA plasmids targeting the human OPN gene sequence were provided in the pLKO.1 plasmid (sequences found on Mission shRNA website; Sigma, St. Louis, MO). Standard protocol was followed for cDNA and RT-PCR using the following primers: OPN, forward TTGCAGCCTTCTCAGCCAA and reverse CAAAAGCAAATCACTGCA ATTCTC, GAPDH, forward GCATGGCCTTCCGTGTCC, reverse AATGCCAGCCCCAGCGTCAAA.

OPN Immunoprecipitation. BJ fibroblasts plated in serum-free for 72 hrs. NETN lysis buffer was added to media (20 mM Tris-HCl (pH 7.9), 1 mM EDTA, 100 mM NaCl, and 0.5% NP40) and incubated overnight at 4°C with OPN antibody (AF1433; R&D, Minneapolis, MN).

Recombinant human OPN (rhOPN). HaCaT cells were plated in a 96 well plate ($1.0x10^3$) and incubated in DME/F12 supplemented with 0.1% FCS for 24 hrs. 100 ng/ml rhOPN (R&D, Minneapolis, MN) was added for 72hrs. N.p.c.T cells were plated

overnight ($1.0x10^3$) and 100ng/ml rhOPN was added in 5% K-sfm/DME/F12 (Invitrogen, Carlsbad, CA) for 72 hrs.

Xenograft model and chemical carcinogenesis protocol. Female NOD/SCID mice (NCI-Fredrick) were housed according to the guidelines of DCM, Washington University School of Medicine. Live *in vivo* imaging as described [26]. Two-step chemical carcinogenesis protocol as previously described [27] on 129S6/SvEv female mice (Taconic, Germantown, NY). To obtain papillomas outbred transgenic mice (*RBP-j*$^{flox/+}$ and *K14CreERT; N1*$^{flox/+}$*; RBP-j*$^{flox/+}$) were treated topically with DMBA (25ug) followed by twice weekly TPA treatments (4ug) for 15 weeks starting one week after DMBA treatment.

Senescent-associated β-galactosidase. Staining was performed on cells and 10um frozen sections as previously described [16].

Immunohistochemistry. Dissected tissue was fixed for 1hr in 4% paraformaldehyde and embedded in paraffin or OCT freezing medium (Sakura Finetek U.S.A, Torrance, CA) and stained with the following antibodies: OPN 1:400 (AF808, R&D, Minneapolis, MN); p16 1:50 (antigen retrieval in pH 9 Tris-buffer; sc-1661, Santa Cruz Biotechnology, Santa Cruz, CA); CD45 1:60 (550539, BD PharMingen, San Diego, CA); F4/80 (MCA497G, Serotec, Raleigh, NC). Detection was visualized with Dako and Vector kits (Dako, Carpinteria, CA and Vector, Burlingame, CA)

Statistical Analysis. Data represent the mean ± SEM; statistical significance was assessed by a two-tailed Student's T-test.

Results

Senescent BJ fibroblasts stimulate the growth of preneoplastic cells *in vitro* and *in vivo*. The tumor microenvironment is an important contributor to neoplastic progression and an increasing body of evidence suggests that it also plays a pivotal role in the early stages of the transformation process. One factor that impacts the stromal compartment is age. Indeed, the presence of senescent cells increases with age [15-17] and these cells possess the capacity to promote preneoplastic cell growth in several models [19, 20, 28-30]. To address the molecular mechanism by which senescent fibroblasts mediate preneoplastic cell growth, we first established a culture system that allowed us to quantitate cell growth *in vitro*. Because the presence of senescent cells within the skin of aging individuals increases with age [15-17] and our microarray analysis (see below) was carried on human skin fibroblasts, we choose to utilize preneoplastic immortalized keratinocytes (HaCaT and N.p.c.T.) for our studies [23, 24]. To facilitate quantification of HaCaT and N.p.c.T. cell growth, we transduced them with click beetle red luciferase (CBR) and grew them in the presence of young or senescent BJ fibroblasts. Senescence can be induced by continued passage leading to replicative senescence (RS), which is believed to be telomere based or by any number of other methods collectively referred to as stress-induced premature senescence (SIPS) [19, 28, 31], all of which have been

shown to stimulate the growth of preneoplastic cells [19, 20, 29, 30]. Therefore, we chose to utilize bleomycin treatment, which reliably induces senescence of BJ skin fibroblasts (**Figure 2.1A**). To confirm that cells undergoing RS and SIPS equivalently stimulated preneoplastic growth, we compared their ability to stimulate the growth of HaCaT cells. As shown in **Figure 2.1B,** RS and SIPS fibroblasts were equally proficient at inducing the growth of HaCaT cells compared to HaCaT cells grown in the presence of young BJ fibroblasts. In addition, live animal luciferase imaging revealed that BJ fibroblasts undergoing RS or SIPS efficiently stimulated the growth of HaCaT xenografts *in vivo* (**Figure 2.1C** and data not shown). Histological analysis of xenografts confirmed that observed by our live animal imaging. Injection of HaCAT cells and senescent fibroblasts resulted in large benign lesions. In contrast, injection of HaCAT cells alone or in the presence of young fibroblasts resulted in small, nearly undetectable lesions (**Figure 1D**).

Analysis of the senescent transcriptome. Given the observed stimulation of HaCaT cell growth after coculture with both RS and SIPS fibroblasts, we next carried out microarray analysis to identify putative senescent-associated candidate genes. Given that fibroblasts undergoing RS or SIPS similarly induced the growth of preneoplastic cells [20] (**Figure 2.1B**), we hypothesized that a common core of genes was responsible for their growth-promoting activities. Therefore, we conducted microarray analysis under conditions that captured the cell culture conditions utilized in our coculture experiments (i.e. serum-free and 3% O_2). Using these conditions, RNA was isolated from BJ skin

fibroblasts that had undergone RS or SIPS following bleomycin treatment and compared the expression profile to young BJ fibroblasts.

Analysis of the resultant microarray data revealed a significant alteration in gene expression in cells undergoing senescence versus their younger counterparts. As expected, gene expression differences were observed between cells undergoing RS versus SIPS. However, a significant overlap in the gene expression patterns of RS and SIPS fibroblasts was apparent (**Figure 2.2A**). Indeed, when comparing genes differentially expressed in each senescent population compared to young fibroblasts, 354 were coordinately regulated in RS and SIPS fibroblasts (**Figure 2.2B**). GO analysis of the data revealed that the coordinately regulated gene groups included proliferative and mitogenic genes, genes involved in extracellular matrix (ECM) functions and inflammation (**Figure 2.2C**). Many of these genes code for secreted growth factors, chemokines, wound repair proteins, and matrix remodeling proteins, which have been implicated in tumor stroma-epithelial interactions [10, 11]. We also found that senescent fibroblasts increased expression of AREG and MMP3, which impact preneoplastic cell growth and morphology, respectively [19, 28]. These observations demonstrate that senescent fibroblasts possess a significantly altered gene expression pattern compared to young fibroblasts and supports the hypothesis that activation of senescence is likely to alter the microenvironment, leading to enhanced preneoplastic cell growth and tumor formation in a manner analogous to cancer associated fibroblasts (CAFs).

To validate our microarray results, we chose a subset of target genes based on their reported secretory and mitogenic properties, reasoning that they would impact

preneoplastic cell growth. Pregnancy-associated plasma protein A (PAPPA) is a metalloprotease that cleaves insulin-like growth factor binding protein 4 (IGFBP4) – an inhibitor of insulin growth factor (IGF) [32]. The direct result of PAPPA's activity is increased bioavailability of IGF, which is a potent growth-regulatory protein. Amphiregulin (AREG) is a member of the epidermal growth factor family that stimulates the growth and proliferation of epithelial cells. It was previously documented that AREG is expressed in senescent fibroblasts and functions in a paracrine fashion to stimulate the growth of prostate epithelial cells [19]. PAI-1, an inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA) was previously shown to mediate induction of senescence [33]. Finally, we examined the expression of osteopontin (OPN), a multifunctional secreted glycoprotein that has been implicated in diverse stages of carcinogenesis (reviewed in [34]). qRT-PCR analysis verified the upregulation pattern observed in the microarray for all of the selected genes (**Figure 2.2D**).

Identification of stromal-derived OPN in preneoplastic lesions coincident with senescent stroma. Our microarray data indicated that OPN was significantly upregulated in senescent fibroblasts, suggesting that it impacts preneoplastic cell growth. OPN was originally identified as a transformation-associated matricellular protein produced by cancer cells [35]. OPN is a secreted protein that is extensively modified by phosphorylation and glycosylation [36] and is targeted by several metalloproteinases [37]. While OPN contributes to mineralization and ECM integrity, it also functions as a potent signaling cytokine [36]. Intriguingly, OPN-null mice display a significant delay in

the appearance and incidence of papillomas in a classical two-stage skin chemical carcinogenesis model [27], suggesting that OPN plays a key role in skin tumorigenesis. To date, studies have focused on the function of epithelial-derived OPN in tumorigenesis and its value as a prognostic marker [38]. However, given the high levels of OPN expressed in senescent fibroblasts (**Figure 2.2**) and the delayed cancer phenotype observed in OPN-null mice [27], we postulated that OPN expressed within the stromal compartment contributes to the transformation process.

Given the possibility that stromal-derived OPN contributes to transformation, we next sought to determine whether senescent stroma was present within a preneoplastic lesion and whether it was coincident with OPN expressing cells. To address this possibility, we took advantage of the two-stage skin carcinogenesis model. We choose to focus on this model because previous studies demonstrated that 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment of murine skin leads to OPN expression [39]. In addition, OPN null mice display a delayed tumor phenotype when treated with this protocol [27]. To determine whether 7,12-dimethylbenz(a)anthracene (DMBA) and/or TPA were sufficient to induce senescence, we first examined skin sections obtained from mice that were treated with a single dose of DMBA followed by three treatments of TPA. We utilized SA-βGal staining to assess the presence of senescent cells. We observed the appearance of SA-βGal positive cells within the stromal compartment of mice treated with DMBA and TPA as well as TPA alone (**Figure 2.3A**). Remarkably, the appearance of senescent stroma precedes the presence of overt hyperplasia as evidenced by the positive staining in TPA only-treated mice (AT) within 7

days of treatment (**Figure 2.3A**). In addition, the same stromal area displayed p16-positive cells, another senescent marker (**Supplemental Figure 2.1**). Using this model, previous work demonstrated the presence of SA-βGal within the epithelial compartment of papillomas that arose several months after the initial DMBA/TPA exposure [40]. While we see very limited SA-βGal staining within the epithelial compartment of the skin, we postulate that the differences observed between our studies is due to the time at which the tissue was analyzed. Indeed, in our hands SA-βGal staining of papillomas arising several months after initial DMBA treatment did reveal limited staining within the epithelial compartment (**Supplemental Figure 2.2**).

We next analyzed tissue from papillomas obtained from mice treated with a single dose of DMBA followed by repeated twice-weekly treatments with TPA, which produces papillomas at predictable latency [41]. As shown in **Figure 2.3C** (upper panel), OPN staining within the stromal compartment of a papilloma coincided with p16 staining while limited staining was observed within the epithelial compartment. In addition, we also observed OPN positive cells in the stromal compartment of DMBA-TPA treated skin adjacent to papillomas (**Figure 2.3C** lower panel). The presence of p16 expression is consistent with the presence of senescent cells (16, 42). SA-βGal staining was also evident within the stromal compartment of the papillomas, further supporting the possibility that the OPN expression was within senescent stromal cells (**Supplemental Figure 2.2**).

Stromal-derived OPN stimulates the growth of preneoplastic cells. Senescent fibroblasts possess growth-promoting activities that stimulate preneoplastic cell growth [19, 20] and our data suggested that OPN functions as an important mediator of this activity. Therefore, to directly assess whether stromal-derived OPN impacted preneoplastic cell growth, BJ fibroblasts were stably transduced with virally encoded RNAi hairpin constructs targeting OPN or a control hairpin construct. We found that knockdown of OPN in senescent fibroblasts by three independent hairpins (shOPN-7, shOPN-8, and shOPN-9) resulted in dramatically reduced mRNA levels (**Figure 2.4A**) and undetectable protein levels (**Figure 2.4B**) when compared to cells transduced with a control hairpin (shSCR). Fibroblasts transduced with hairpins targeting OPN entered senescence upon bleomycin treatment at the same rate as observed in control cells, indicating that OPN expression is not necessary for induction of senescence (**Figure 2.4C**).

Having established that our RNAi constructs efficiently reduced OPN to undetectable levels without impacting the induction of senescence, we next investigated how loss of OPN impacted the growth-promoting properties of senescent fibroblasts. Senescent fibroblasts mock transduced or transduced with a control hairpin (shSCR) increased the growth of HaCaT and N.p.c.T cells compared to cells grown on young fibroblasts (**Figure 2.4D**). In contrast, RNAi directed knockdown of OPN in senescent fibroblasts resulted in a significant reduction in HaCaT and N.p.c.T cell growth (**Figure 2.4D**). Indeed, we found that loss of OPN resulted in HaCaT and N.p.c.T cell growth

similar to that observed when these cells were plated on young fibroblasts, indicating that OPN is necessary for stimulation of preneoplastic cell growth by senescent fibroblasts.

Stromal-derived OPN stimulates the growth of preneoplastic cells in xenografts. Loss of OPN significantly reduced the growth promoting properties of senescent fibroblasts *in vitro*. Therefore, we next addressed the impact of loss of stromal derived OPN on the growth of HaCaT cells in xenografts. Utilizing live *in vivo* luciferase imaging, we found that injection of HaCaT cells alone or in combination with young fibroblasts resulted in little growth of xenografts. In contrast, coinjection of senescent fibroblasts and HaCaT cells resulted in robust growth *in vivo* (**Figure 2.5A**). Similarly, senescent fibroblasts expressing a control hairpin (shSCR) significantly stimulated HaCaT cell growth. In contrast, OPN depletion abolished HaCaT cell growth indicating that OPN derived from senescent fibroblasts is necessary *in vivo* (**Figure 2.5A**). Immunohistological analysis of CD45 and F4/80 in xenografts revealed a decrease in immune infiltration in lesions resulting from injection of senescent cells expressing a hairpin targeting OPN versus a control hairpin (**Figure 2.5B).** Together, these experiments indicate that stromal-derived OPN impacts the microenvironment and significantly stimulates preneoplastic cell growth.

rhOPN is sufficient to stimulate the growth of preneoplastic cells. The above experiments demonstrate that stromal-derived OPN significantly impacts preneoplastic cell growth. We next sought to determine whether OPN is sufficient to promote

preneoplastic cell growth. To address this question, we treated preneoplastic cells (HaCaT/N.p.c.T) with recombinant human OPN (rhOPN) and measured its impact on cell growth. We found that addition of rhOPN to both HaCaT and N.p.c.T cells conferred a modest but significant growth advantage compared to mock-treated cells, indicating that OPN acts as a paracrine factor that is sufficient for preneoplastic cell growth (**Figure 2.6**). The increased growth observed upon rhOPN treatment was less robust then when HaCaT or N.p.c.T cells are plated directly on senescent fibroblasts expressing OPN (**Figure 2.4**). Because senescent fibroblasts express numerous secretory proteins it is possible that the reduced response observed upon rhOPN treatment reveals a requirement for additional factors. Alternatively, the reduced response could be attributed to a lack of adequate protein modifications on the rhOPN protein. As stated earlier, OPN is a highly modified protein [42] and careful inspection of a longer exposure of our western blot (**Supplemental Figure 2.3**) revealed the presence of additional OPN species in supernatants obtained from senescent fibroblasts that were not present in the rhOPN preparations.

Discussion

Alterations in the tissue microenvironment in combination with cell autonomous mutations collaborate to drive the transformation process. Here, we identify osteopontin (OPN) as a critical stromal-derived mediator of preneoplastic cell growth. Utilizing microarray analysis we found that OPN is upregulated in senescent fibroblasts while being undetectable in their younger counterparts. We found that senescent fibroblast-

derived OPN was necessary for preneoplastic cell growth *in vitro* and *in vivo*. In addition, recombinant OPN was sufficient to stimulate the growth of preneoplastic cells. Finally, examination of papillomas that arose following treatment of mice with a DMBA/TPA protocol revealed the presence of senescent stromal cells and OPN expression within the stromal compartment. Taken together, our data argue that senescent fibroblast-derived OPN contributes to tumorigenesis and reveal a previously uncharacterized stromal function for OPN that may represent a novel therapeutic target and/or biomarker for the early stages of cancer development.

Investigations into the cell types within the microenvironment that impact tumorigenesis have revealed that macrophages, B and T cells, mast cells, adipocytes, endothelial cells, and fibroblasts can affect primary growth and metastasis. Cancer associated fibroblasts (CAF) exert their influence on neoplastic progression through several mechanisms such as secretion of paracrine factors that directly stimulate cancer cells and impact proliferation and survival. While senescent fibroblasts differ from CAFs in regards to their proliferative capacity, they possess the ability to stimulate preneoplastic and neoplastic cell growth in a manner analogous to CAFs [19, 20]. This latter observation raises the possibility that the accumulation of senescent cells within aged individuals alters the tissue microenvironment that in turn collaborates with cell autonomous mutations to drive the transformation process. Intriguingly, we show that senescent stroma appears before the emergence of a papilloma, arguing that stromal changes arise early in the transformation process. In support of this hypothesis, our microarray data indicate that in addition to OPN, senescent fibroblasts express numerous

factors capable of remodeling the microenvironment and modulating cell growth in a paracrine fashion. We propose that these early changes, which include the appearance of senescent stroma and concomitant upregulation of OPN, provide a rich microenvironment for tumorigenesis.

OPN is a pleiotropic protein to which numerous functions have been ascribed including mineralization and calcification, osteoclast activation, endothelial cell survival, wound healing, migration, lymphocyte proliferation and immunosurveillance [34]. Interestingly, in human cancer, OPN expression levels correlate with tumor grade and elevated OPN serum levels is a poor prognostic indicator in a wide variety of tumors [34]. Murine studies revealed that OPN null mice display a significant delay in tumorigenesis when treated with the classic two-step skin carcinogenesis model of DMBA/TPA treatment [27], suggesting that OPN plays a key role in cellular transformation.

To date, investigation into OPN's role in tumorigenesis has been limited to the impact of epithelial-derived OPN on transformation and progression. However, a functional role for stromal-derived OPN in tumorigenesis has not been investigated even though other cell types including macrophages and neutrophils express OPN under both normal and pathological conditions [43]. Notably, a recent microarray analysis of the stromal compartment of human breast cancers identified osteopontin as one of twenty-six genes that when overexpressed is a strong predictor of clinical outcome [44]. Our results showing increased OPN in the stromal compartment of DMBA/TPA treated mice suggest an even more general role for OPN in epithelial tumorigenesis. Together, these

observations indicate that non-epithelial derived OPN is present at various stages of tumorigenesis in human cancer as well as mouse models, and support a physiologic role for this source of OPN in transformation.

OPN is clearly important for the growth promoting effects of senescent fibroblasts as OPN depletion from senescent fibroblasts drastically reduces growth of preneoplastic cells. However, our data indicate that recombinant human OPN (rhOPN) does not fully mimic the enhanced preneoplastic cell growth observed when cells are grown in the presence of senescent fibroblasts expressing OPN. The inability of rhOPN to recapitulate the magnitude of preneoplastic cell growth observed in our coculture conditions raises the possibility that other factors cooperate with fibroblast-derived OPN. Alternatively, posttranslational modifications of stromal-derived OPN may be important. OPN is a heavily modified protein that possesses metalloproteinases (MMP) cleavage sites [37]. Both protein modifications and cleavage impact OPN function [45, 46]. Interestingly, analysis of our microarray data indicated that several MMPs are expressed at high levels in senescent fibroblasts. Western blot analysis of conditioned medium obtained from senescent fibroblasts revealed the presence of OPN species that were not present in our rhOPN preparation, raising the possibility that one or more of these species mediates the growth-promoting activities of senescent fibroblasts. In addition, despite the high levels of OPN mRNA present within senescent fibroblasts, we did not detect the protein in the cell pellets but instead were only able to detect OPN in the conditioned media of senescent fibroblasts, indicating that a significant amount of the protein is present as a

soluble factor within the extracellular space. Such data suggests that the presence of OPN in the stroma of premalignant lesions such as papillomas may be underappreciated.

Our data and that of others [19, 20] support a role for senescent stroma in preneoplastic lesions but does not preclude its involvement in the later stages of transformation and cancer development. Interrogation of our senescent microarray data revealed a prominent inflammatory profile, which could facilitate recruitment of other components of the microenvironment in addition to impacting the ECM, bone marrow cell recruitment, and angiogenesis. Intriguingly, CAFs also express many of these factors, some of which are able to promote vascularization by recruiting endothelial cells [47]. Furthermore, a recent report demonstrates that CAFs secrete extracellular matrix tracks on which tumor cells can migrate, thus facilitating metastasis [48]. While OPN has been shown to be important for metastasis and is a validated prognostic marker in human neoplasias, it was recently shown to function as a systemic instigator by mobilizing and recruiting bone marrow-derived cells to the tumor stroma [49]. Interestingly, immunohistochemical analysis of xenografts arising from injections with stromal cells in the absence of OPN revealed a decrease in immune infiltration compared to those arising in the presence of OPN expressing stromal cells. This observation raises the possibility that senescent stromal-derived OPN affects recruitment of cells of the innate immune system potentially through bone marrow mobilization. These results underscore the importance of paracrine signaling and the interplay between different components of the tumor microenvironment at multiple stages of transformation.

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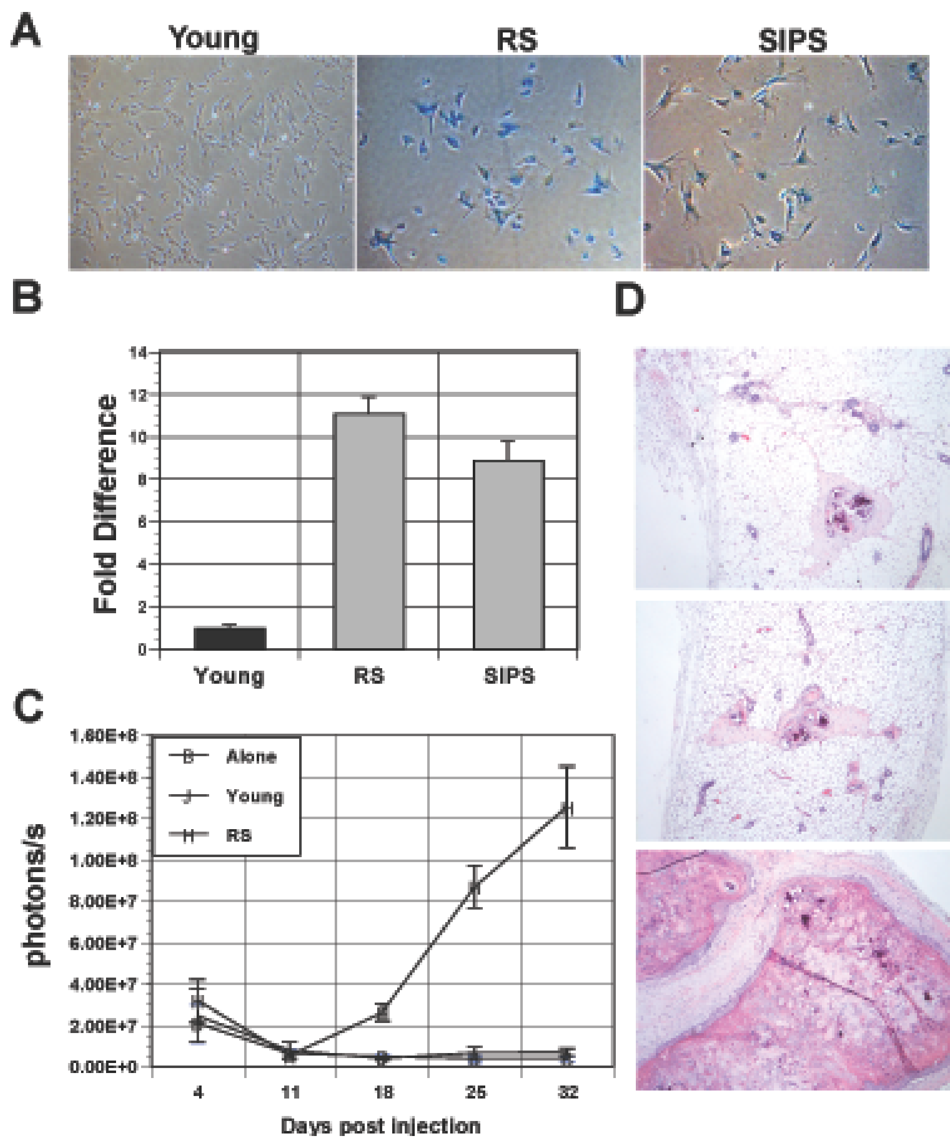


Figure 2.1 Senescent fibroblasts stimulate the growth of preneoplastic cells. A, continued passage (RS) and treatment with 100 ug/ml bleomycin (SIPS) induces robust senescence characterized by induction of senescence associated β–galactosidase (SA-βGal) staining. Magnification 20X. B, fibroblasts undergoing RS or SIPS stimulate the *in vitro* growth of HaCaT keratinocytes expressing click beetle red luciferase. HaCaT cells were plated on lawns of young, RS, or SIPS fibroblasts and their growth was measured by relative luciferase activity. Wells containing HaCAT cells and young fibroblasts were set to 1. Fold increase in growth over wells with young fibroblasts is plotted. $p<0.05$. C, HaCAT cells were injected alone or with young or senescent

fibroblasts (RS) into NOD/SCID mice. For each injection, 2.5×10^5 HaCaT cells were coinjected with 7.5×10^5 fibroblasts. *In vivo* imaging of luciferase activity was used to assess HaCaT cell growth weekly. Each line represents the average photons/sec for 6 independent injection sites. Student's t test at day 28, $p < 0.05$. D, Hematoxylin and eosin stains of histological sections obtained from xenografts injected with HaCaT cells alone or in the presence of young or senescent fibroblasts (top to bottom). Magnification 10X.

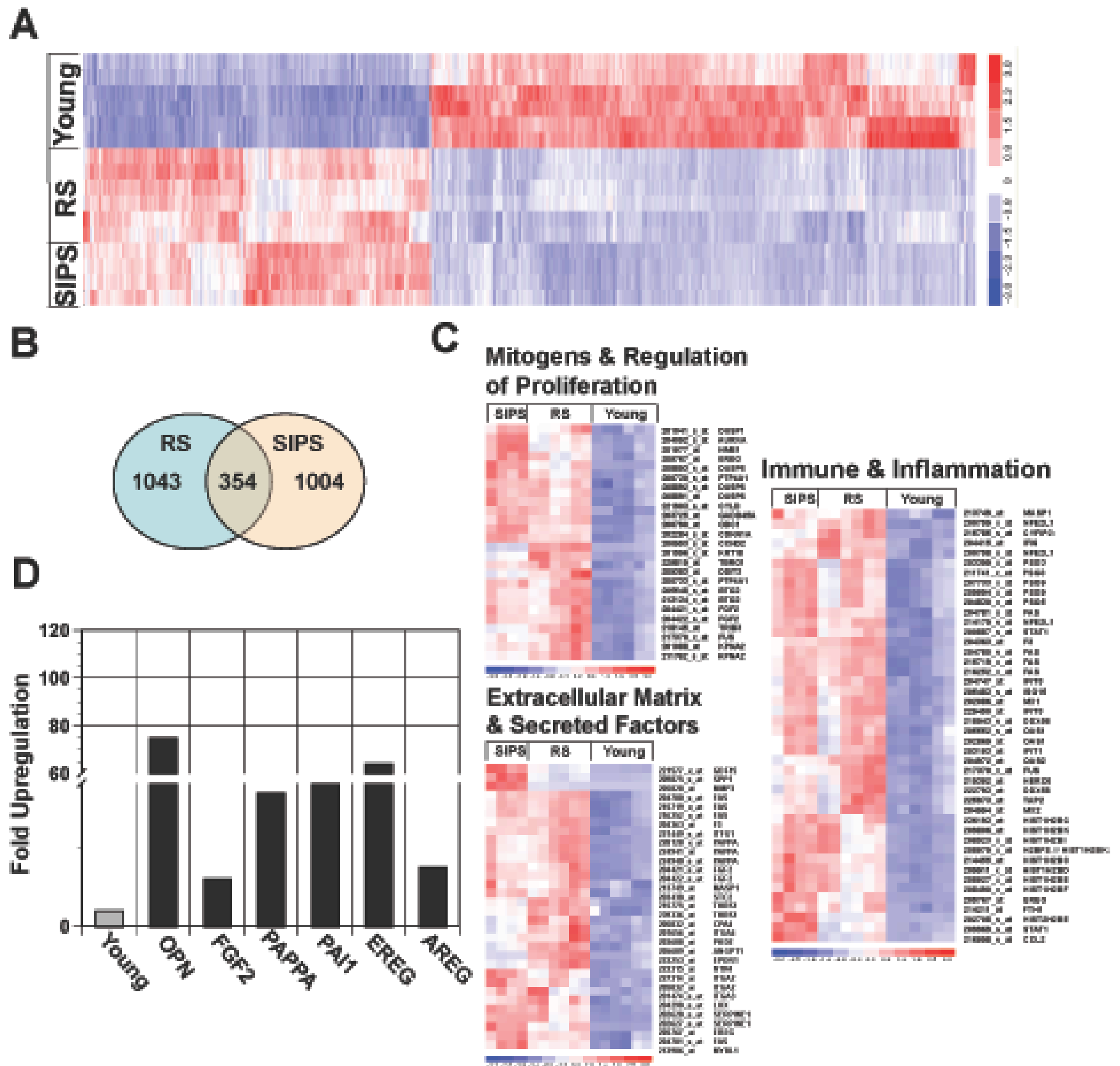


Figure 2.2 Analysis of the senescence transriptome reveals significant overlap between cells undergoing replicative and stress-induced premature senescence. A, microarray analysis of young BJ fibroblasts and BJ fibroblasts undergoing RS or bleomycin induced SIPS. Hierarchical clustering of consistent transcript alterations across all conditions and replicas are plotted. B, Venn diagram plots genes that significantly overlap in fibroblasts undergoing RS and SIPS versus young. C, Gene ontology analysis (GO) of upregulated classes in cells undergoing RS and SIPS compared to their younger counterparts (i.e. Immune & Inflammation, Mitogens & Regulation of Proliferation, Extracellular Matrix & Secreted Factors). D, Fold upregulation of select genes in senescent fibroblasts (SIPS) was confirmed by qRT-PCR. Expression in young fibroblasts was set as one for all genes.

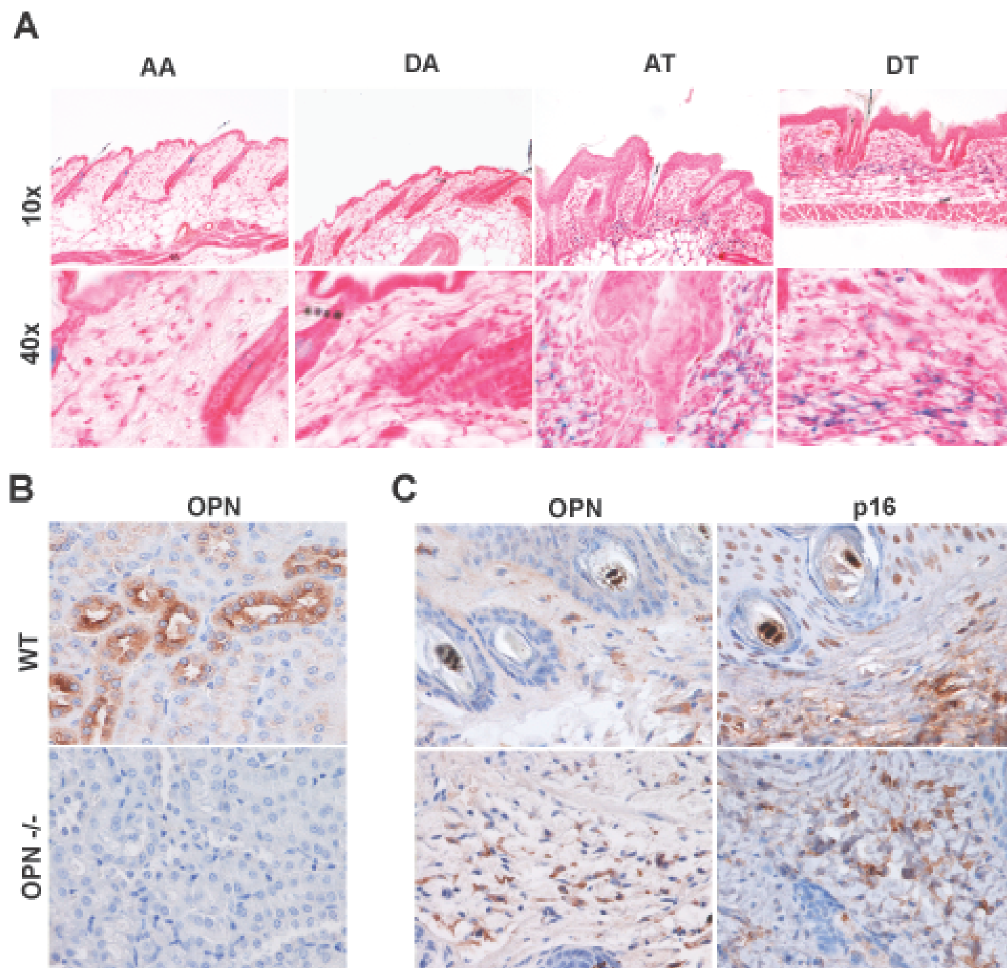


Figure 2.3 Senescent stroma is present in preneoplastic lesions following DMBA-TPA treatment. A, Representative SA-βGal staining of frozen tissue sections reveal robust staining within the stromal compartment (n=3). Skin treatments are as follows: (1) AA, acetone followed by weekly acetone treatment (2) DA, DMBA followed by weekly acetone treatment; (3) AT, acetone followed by weekly TPA treatment; (4) DT, DMBA followed by weekly TPA treatments. B, Immunohistochemical analysis of osteopontin (OPN) in kidney sections from wild type (C57/B6) and OPN deficient mice. Magnification upper panel 10X and lower panel 40X. C, OPN and p16 expression in DMBA/TPA induced papillomas (upper panel) and associated skin (lower panel). Serial paraffin embedded tissue sections were stained with antibodies against OPN and the cell cycle inhibitor p16. Magnification 40X. (n=2 of 6).

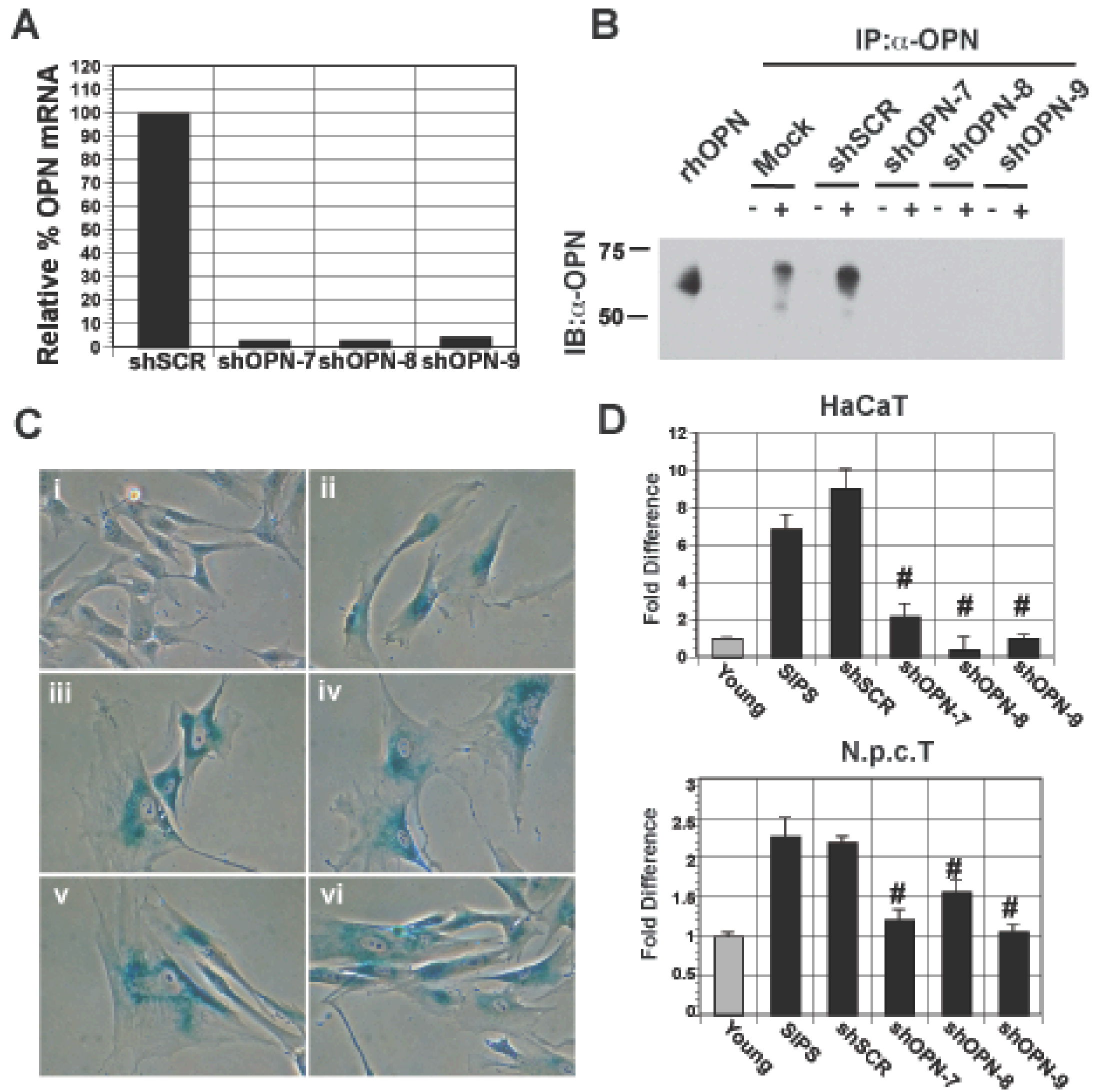


Figure 2.4 Senescent-derived OPN is necessary for preneoplastic cell growth. A, Stable knockdown of OPN expression by viral-based RNAi is revealed by qRT-PCR. OPN mRNA expression in senescent fibroblasts transduced with a control hairpin (shSCR) was set to 100%. Three independent short hairpins targeting OPN (shOPN-7, -8, -9) resulted in robust knockdown of OPN mRNA. B, Western blot analysis (IB) of OPN immunoprecipitations (IP) from cultured supernatants obtained from young (-) or senescent (+) fibroblasts mock transduced, transduced with a control hairpin (shSCR), or transduced with hairpins targeting OPN (shOPN-7, -8, -9). Recombinant human OPN

(rhOPN) is included as a reference. C, RNAi directed loss of OPN does not affect the induction of senescence upon bleomycin treatment. SA-βGal expression in i) young, ii) SIPS, iii) shSCR SIPS, iv) shOPN-7 SIPS, v) shOPN-8 SIPS, and vi) shOPN-9 SIPS BJ fibroblasts. Magnification 40X. D, Upper Panel: Quantification of HaCAT cell growth when plated on lawns of fibroblasts. Preneoplastic cell growth was measured by relative luciferase activity and wells where HaCAT cells were plated with young BJ fibroblasts were set to 1. OPN expression was depleted in senescent BJ fibroblasts by one of three independent hairpins (shOPN-7, -8, -9) to greater than 95% in all cases. A control hairpin (shSCR) was used as a negative control. Depletion of OPN expression reduced preneoplastic cell growth to levels observed in cells plated with young fibroblasts. #$p<0.05$. Lower Panel: Quantification of N.p.c.T cell growth when plated on lawns of fibroblasts. Loss of OPN expression (shOPN-7, -8, -9) resulted in reduced growth similar to that observed with HaCaT cells shown in upper panel. #$p<0.05$.

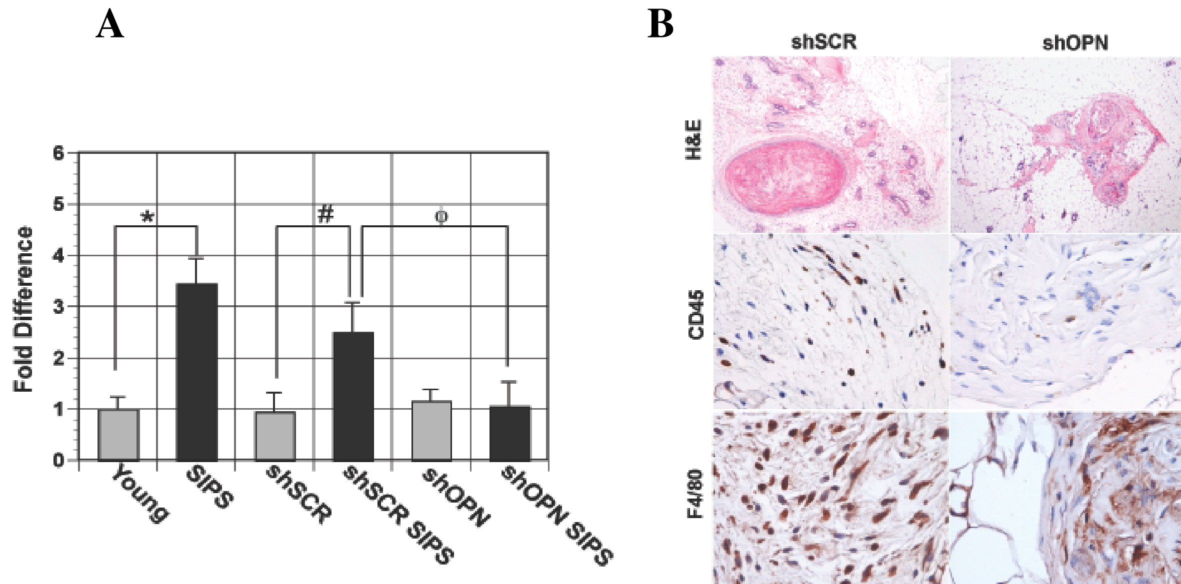


Figure 2.5 Loss of OPN expression in senescent fibroblasts results in reduced preneoplastic cell growth *in vivo*. Fibroblasts were uninfected (young and SIPS), infected with a control virus (shSCR, sSCR SIPS are young and senescent fibroblasts, respectively), or a virus that knocks down OPN expression (shOPN, shOPN SIPS are young and senescent fibroblasts, respectively) greater than 95%. Relative to SIPS fibroblasts, knockdown of OPN resulted in a significant reduction in growth of HaCaT xenografts in NOD/SCID mice (n=3). *p=0.003, $^{\#}$p=0.04, and $^{\Phi}$p= 0.06. B, Immunohistochemical analysis of xenografts obtained from mice injected with HaCaT cells and senescent fibroblasts expressing a control hairpin (shSCR) (upper panel) or a hairpin targeting OPN (shOPN) (lower panel). Shown are Hematoxylin and Eosin (H&E) and staining for the immune markers CD45 and F4/80, which identify leukocytes and macrophages, respectively. Photos focus on stromal sections showing CD45 or F4/80 positive staining. Magnification for H&E, 10X and for CD45 and F4/80, 40X.

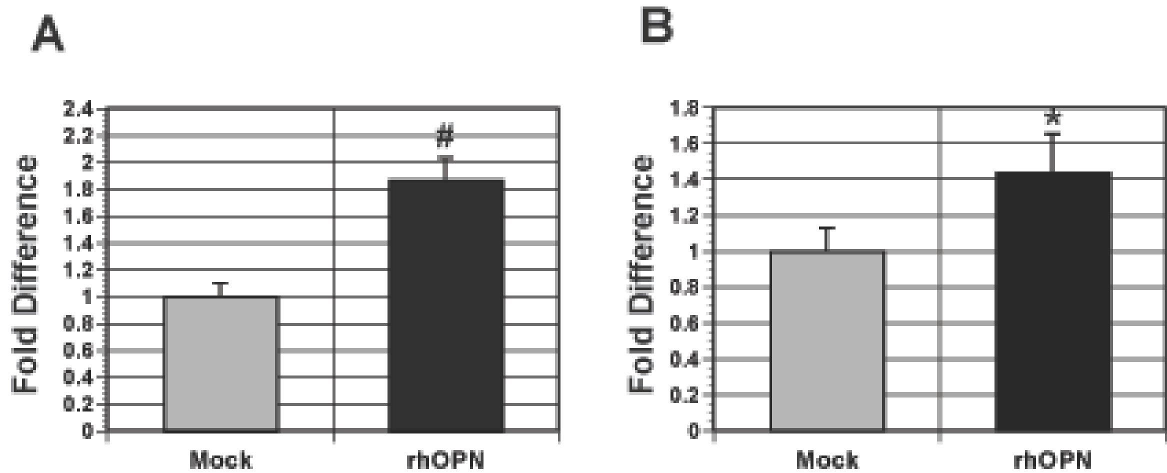
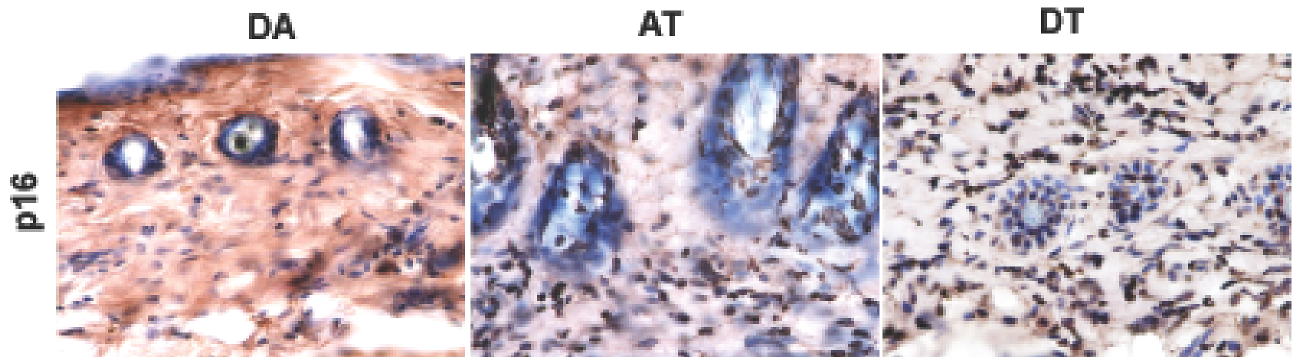
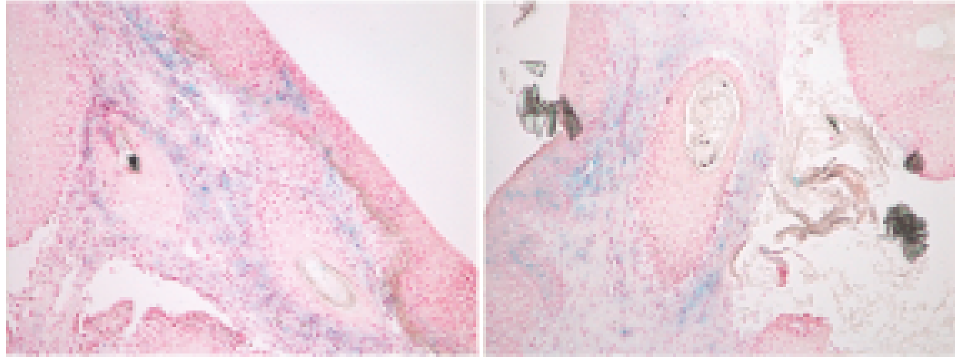


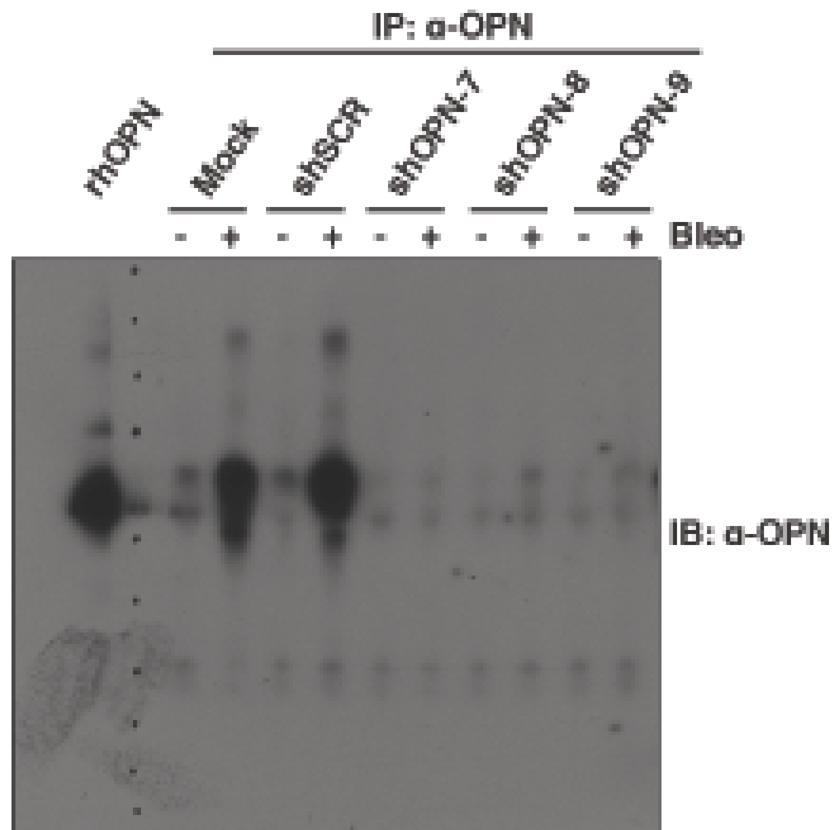
Figure 2.6 OPN is sufficient to stimulate the growth of preneoplastic cells. Cell growth was measured by relative luciferase activity and the growth of cells receiving BSA alone was set to 1. A, addition of 100 ng/ml of rhOPN was sufficient to stimulate the growth of HaCaT cells. #p<0.05. B, addition of 100 ng/ml of rhOPN was sufficient to stimulate the growth of N.p.c.T. cells. *p=0.05.



Supplemental Figure S2.1 p16 positive cells are present in the stromal compartment following treatment with TPA. Immunohistochemistry in skin sections from mice treated with DMBA or TPA alone, or with DMBA-TPA. Mice were treated with DMBA followed by weekly acetone treatment (DA), acetone followed by weekly TPA treatment (AT), or DMBA followed by weekly TPA treatments (TA). Frozen skin sections (exposed to the same treatment as shown on Figure 3) were stained for p16 – a senescent marker. Magnification 40X.



Supplemental Figure S2.2 Senescent stromal cells are present within papillomas that arise following DMBA-TPA treatment. SA-βGal staining of frozen tissue sections obtained from papillomas reveals the presence of senescent cells within the stromal compartment. Magnification 20X.



Supplemental Figure S2.3 Multiple OPN species are found in conditioned medium obtained from senescent fibroblasts. The gel is a longer exposure of the gel pictured in **Figure 4** that reveals the presence of at least two unique OPN species that are not present in the rhOPN preparation.

References

1. DePinho, R.A. (2000). The age of cancer. Nature *408*, 248-254.

2. Sakr, W.A., Haas, G.P., Cassin, B.F., Pontes, J.E., and Crissman, J.D. (1993). The frequency of carcinoma and intraepithelial neoplasia of the prostate in young male patients. J Urol *150*, 379-385.

3. Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999). Creation of human tumor cells with defined genetic elements. Nature *400*, 464-468.

4. Hahn, W.C., and Meyerson, M. (2001). Telomerase activation, cellular immortalization and cancer. Ann Med *33*, 123-129.

5. Zhao, J.J., Roberts, T.M., and Hahn, W.C. (2004). Functional genetics and experimental models of human cancer. Trends Mol Med *10*, 344-350.

6. Martins-Green, M., Boudreau, N., and Bissell, M.J. (1994). Inflammation is responsible for the development of wound-induced tumors in chickens infected with Rous sarcoma virus. Cancer Res *54*, 4334-4341.

7. de Visser, K.E., Korets, L.V., and Coussens, L.M. (2005). De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. Cancer Cell *7*, 411-423.

8. Peek, R.M., Jr., and Crabtree, J.E. (2006). Helicobacter infection and gastric neoplasia. J Pathol *208*, 233-248.

9. Greenson, J.K. (2002). Dysplasia in inflammatory bowel disease. Semin Diagn Pathol *19*, 31-37.

10. Tuxhorn, J.A., Ayala, G.E., and Rowley, D.R. (2001). Reactive stroma in prostate cancer progression. J Urol *166*, 2472-2483.

11. Bissell, M.J., and Radisky, D. (2001). Putting tumours in context. Nat Rev Cancer *1*, 46-54.

12. Olumi, A.F., Grossfeld, G.D., Hayward, S.W., Carroll, P.R., Tlsty, T.D., and Cunha, G.R. (1999). Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res *59*, 5002-5011.

13. Nielsen, M., Thomsen, J.L., Primdahl, S., Dyreborg, U., and Andersen, J.A. (1987). Breast cancer and atypia among young and middle-aged women: a study of 110 medicolegal autopsies. Br J Cancer *56*, 814-819.

14. Bhathal, P.S., Brown, R.W., Lesueur, G.C., and Russell, I.S. (1985). Frequency of benign and malignant breast lesions in 207 consecutive autopsies in Australian women. Br J Cancer *51*, 271-278.

15. Castro, P., Giri, D., Lamb, D., and Ittmann, M. (2003). Cellular senescence in the pathogenesis of benign prostatic hyperplasia. Prostate *55*, 30-38.

16. Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., and et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A *92*, 9363-9367.

17. Herbig, U., Ferreira, M., Condel, L., Carey, D., and Sedivy, J.M. (2006). Cellular senescence in aging primates. Science *311*, 1257.

18. Trougakos, I.P., Saridaki, A., Panayotou, G., and Gonos, E.S. (2006). Identification of differentially expressed proteins in senescent human embryonic fibroblasts. Mech Ageing Dev *127*, 88-92.

19. Bavik, C., Coleman, I., Dean, J.P., Knudsen, B., Plymate, S., and Nelson, P.S. (2006). The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. Cancer Res *66*, 794-802.

20. Krtolica, A., Parrinello, S., Lockett, S., Desprez, P.Y., and Campisi, J. (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci U S A *98*, 12072-12077.

21. Liu, D., and Hornsby, P.J. (2007). Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. Cancer Res *67*, 3117-3126.

22. Saharia, A., Guittat, L., Crocker, S., Lim, A., Steffen, M., Kulkarni, S., and Stewart, S.A. (2008). Flap endonuclease 1 contributes to telomere stability. Curr Biol *18*, 496-500.

23. Boukamp, P., Petrussevska, R.T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N.E. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol *106*, 761-771.

24. Rheinwald, J.G., Hahn, W.C., Ramsey, M.R., Wu, J.Y., Guo, Z., Tsao, H., De Luca, M., Catricala, C., and O'Toole, K.M. (2002). A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. Mol Cell Biol *22*, 5157-5172.

25. Li, C., and Wong, W.H. (2001). Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci U S A *98*, 31-36.

26. Luker, K.E., Smith, M.C., Luker, G.D., Gammon, S.T., Piwnica-Worms, H., and Piwnica-Worms, D. (2004). Kinetics of regulated protein-protein interactions revealed with firefly luciferase complementation imaging in cells and living animals. Proc Natl Acad Sci U S A *101*, 12288-12293.

27. Hsieh, Y.H., Juliana, M.M., Hicks, P.H., Feng, G., Elmets, C., Liaw, L., and Chang, P.L. (2006). Papilloma development is delayed in osteopontin-null mice: implicating an antiapoptosis role for osteopontin. Cancer Res *66*, 7119-7127.

28. Parrinello, S., Coppe, J.P., Krtolica, A., and Campisi, J. (2005). Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. J Cell Sci *118*, 485-496.

29. Yang, G., Rosen, D.G., Zhang, Z., Bast, R.C., Jr., Mills, G.B., Colacino, J.A., Mercado-Uribe, I., and Liu, J. (2006). The chemokine growth-regulated oncogene 1 (Gro-1) links RAS signaling to the senescence of stromal fibroblasts and ovarian tumorigenesis. Proc Natl Acad Sci U S A *103*, 16472-16477.

30. Kang, J., Chen, W., Xia, J., Li, Y., Yang, B., Chen, B., Sun, W., Song, X., Xiang, W., Wang, X., Wang, F., Bi, Z., and Wan, Y. (2008). Extracellular matrix secreted by senescent fibroblasts induced by UVB promotes cell proliferation in HaCaT cells through PI3K/AKT and ERK signaling pathways. Int J Mol Med *21*, 777-784.

31. Aoshiba, K., Tsuji, T., and Nagai, A. (2003). Bleomycin induces cellular senescence in alveolar epithelial cells. Eur Respir J *22*, 436-443.

32. Conover, C.A., Bale, L.K., Overgaard, M.T., Johnstone, E.W., Laursen, U.H., Fuchtbauer, E.M., Oxvig, C., and van Deursen, J. (2004). Metalloproteinase pregnancy-associated plasma protein A is a critical growth regulatory factor during fetal development. Development *131*, 1187-1194.

33. Kortlever, R.M., Higgins, P.J., and Bernards, R. (2006). Plasminogen activator inhibitor-1 is a critical downstream target of p53 in the induction of replicative senescence. Nat Cell Biol *8*, 877-884.

34. Rangaswami, H., Bulbule, A., and Kundu, G.C. (2006). Osteopontin: role in cell signaling and cancer progression. Trends Cell Biol *16*, 79-87.

35. Senger, D.R., Wirth, D.F., and Hynes, R.O. (1979). Transformed mammalian cells secrete specific proteins and phosphoproteins. Cell *16*, 885-893.

36. Wai, P.Y., and Kuo, P.C. (2004). The role of Osteopontin in tumor metastasis. J Surg Res *121*, 228-241.

37. Agnihotri, R., Crawford, H.C., Haro, H., Matrisian, L.M., Havrda, M.C., and Liaw, L. (2001). Osteopontin, a novel substrate for matrix metalloproteinase-3 (stromelysin-1) and matrix metalloproteinase-7 (matrilysin). J Biol Chem *276*, 28261-28267.

38. Fedarko, N.S., Jain, A., Karadag, A., Van Eman, M.R., and Fisher, L.W. (2001). Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. Clin Cancer Res *7*, 4060-4066.

39. Craig, A.M., Bowden, G.T., Chambers, A.F., Spearman, M.A., Greenberg, A.H., Wright, J.A., McLeod, M., and Denhardt, D.T. (1990). Secreted phosphoprotein mRNA is induced during multi-stage carcinogenesis in mouse skin and correlates with the metastatic potential of murine fibroblasts. Int J Cancer *46*, 133-137.

40. Sun, P., Yoshizuka, N., New, L., Moser, B.A., Li, Y., Liao, R., Xie, C., Chen, J., Deng, Q., Yamout, M., Dong, M.Q., Frangou, C.G., Yates, J.R., 3rd, Wright, P.E., and Han, J. (2007). PRAK is essential for ras-induced senescence and tumor suppression. Cell *128*, 295-308.

41. Yuspa, S.H. (1998). The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis. J Dermatol Sci *17*, 1-7.

42. Rittling, S.R., and Chambers, A.F. (2004). Role of osteopontin in tumour progression. Br J Cancer *90*, 1877-1881.

43. Denhardt, D.T., Noda, M., O'Regan, A.W., Pavlin, D., and Berman, J.S. (2001). Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. J Clin Invest *107*, 1055-1061.

44. Finak, G., Bertos, N., Pepin, F., Sadekova, S., Souleimanova, M., Zhao, H., Chen, H., Omeroglu, G., Meterissian, S., Omeroglu, A., Hallett, M., and Park, M. (2008). Stromal gene expression predicts clinical outcome in breast cancer. Nat Med *14*, 518-527.

45. Christensen, B., Kazanecki, C.C., Petersen, T.E., Rittling, S.R., Denhardt, D.T., and Sorensen, E.S. (2007). Cell type-specific post-translational modifications of mouse osteopontin are associated with different adhesive properties. J Biol Chem *282*, 19463-19472.

46. Kazanecki, C.C., Uzwiak, D.J., and Denhardt, D.T. (2007). Control of osteopontin signaling and function by post-translational phosphorylation and protein folding. J Cell Biochem *102*, 912-924.

47. Orimo, A., Gupta, P.B., Sgroi, D.C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., Carey, V.J., Richardson, A.L., and Weinberg, R.A. (2005). Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell *121*, 335-348.

48. Gaggioli, C., Hooper, S., Hidalgo-Carcedo, C., Grosse, R., Marshall, J.F., Harrington, K., and Sahai, E. (2007). Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat Cell Biol *9*, 1392-1400.

49. McAllister, S.S., Gifford, A.M., Greiner, A.L., Kelleher, S.P., Saelzler, M.P., Ince, T.A., Reinhardt, F., Harris, L.N., Hylander, B.L., Repasky, E.A., and Weinberg, R.A. (2008). Systemic endocrine instigation of indolent tumor growth requires osteopontin. Cell *133*, 994-1005.

Chapter 3: Transcriptional Regulation of Senescent Stromal-Derived Osteopontin

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E.Pazolli was the principal contributor to this work.

Abstract

Senescent fibroblasts stimulate preneoplastic cell growth in a paracrine fashion due to an altered secretory profile enriched in growth factors, cytokines, and extracellular-remodeling enzymes that are regulated at the transcriptional level by mechanisms not yet clearly defined. This expression pattern has been referred to as the senescence-associated secretory phenotype (SASP). Here we demonstrate that the regulation of one of these key molecules, osteopontin (OPN), is unaffected by the inhibition of p53, Rb, and NfκB and independent of the senescence-associated DNA damage response. To identify putative regulators of OPN, I screened several fragments of the human OPN promoter for maximum activity in senescent fibroblasts. We identify a responsive region that contains a canonical Myb binding site and other recognition motifs. Based on this sequence, I conducted an RNAi screen, which revealed several factors that impacted OPN transcription. Because chromatin modulation has been implicated in senescence regulation, I also included several chromatin-modifying genes in the screen. Based on the results from this screen I propose that changes in chromatin structure directly stimulate SASP activation. Indeed treatment with agents that induce chromatin relaxation in the absence of overt DNA damage induces a robust SASP. However, OPN in contrast to two other members of SASP, IL6 and IL8, does not require ATM signaling nor NfκB activation. These findings indicate that components of the secretory profile, while coordinately upregulated, are controlled by distinct transcriptional pathways.

Introduction

Senescence is a permanent cell cycle arrest that is most often characterized by a flattened morphology, increased senescence-associated β-galactosidase (SA-βgal) activity and the appearance of facultative heterochromatin domains known as senescence-associated heterochromatin foci (SAHFs) [1]. While growth-arrested, senescent cells remain metabolically active. In addition, extensive microarray analysis has revealed that senescent cells display a programmatic change in their transcriptional profile characterized by a pronounced inflammatory signature, increased production of growth factors, and enhanced secretion of extracellular matrix (ECM) remodeling enzymes. Because a large percent of the proteins modulated upon the activation of senescence represent secretory proteins, this program has been referred to as the senescence-associated secretory pathway (SASP) [2].

Work from several groups in recent years has demonstrated that such gene expression alterations not only enforce the cell cycle arrest in the originating cell but impact the surrounding milieu as well [3-5]. Similar to cancer-associated fibroblasts, senescent fibroblasts modulate the tumor microenvironment in several ways: secreted mitogens stimulate premalignant epithelial cell growth *in vitro* and in xenograft models [6, Bavik, 2006 #385, 7-9], ECM remodeling enzymes such as matrix metalloproteinases affect branching and migration [10] and other factors including cytokines promote invasion [11, 12]. The ability of senescent fibroblasts to influence tumorigenesis has

been documented convincingly in multiple systems; however, until recently, the underlying mechanisms governing SASP in senescent fibroblasts were unknown.

One of the features of the senescent transcriptome is a pronounced inflammatory signature [5, 13]. Members of this inflammatory core such as IL6, IL8, and CXCR2 are required for the induction and maintenance of senescence in addition to acting as paracrine effectors of tumor growth. Regulation of such chemokines in other settings by NfκB and C/EBβ appears to operate in senescence as well. In fact, both transcription factors occupy promoters of IL6 as well as other cytokines *in vivo* [4, 5]. RNAi-directed silencing leads to abrogation of senescence and its associated transcriptome indicating that IL6 enforces a signaling cascade that activates the senescence program [5]. This is not the first instance that these transcription factors have been linked to senescence. Comprehensive biocomputational analysis of several murine tissues demonstrated that the NfκB motif is one of the transcriptional modules most highly associated with ageing [14]. Likewise, C/EBPβ cooperates with E2F to execute permanent arrest in mouse embryonic fibroblasts [15].

The question remains how these transcription factors are activated in response to senescence stimuli and subsequently directed to activate the transcriptional changes occurring in senescence. It is conceivable that the DNA damage response (DDR), which has been closely linked and possibly contributes to senescence [16, 17] triggers the signaling cascade. Indeed, recent reports argue that a persistent DNA damage response such as the one observed in senescence [17] is sufficient to activate the secretory

phenotype. Signaling downstream of ATM (including NBS1 and Chk2) appears to control a subset of the secreted factors, represented by IL6 and IL8 [11]. These results are intriguing; however, forced expression of a cell cycle inhibitor induces senescence and a paracrine response that stimulates preneoplastic growth in the absence of DNA damage [6] indicating that DNA damage is not a prerequisite for SASP activation.

Activation of DDR leads to chromatin alterations that can have a profound impact on numerous transcriptional pathways. Therefore, it is possible that the transcriptional changes observed in senescent cells result from global chromatin modulation. There are certainly several pieces of evidence that implicate chromatin remodeling in the establishment of the senescent state. For example, several studies have reported the appearance of SAHFs in senescent cells and shown that they occur at E2F promoters and functionally repress proliferation [18-21]. Conversely, senescence is associated with transcriptional activation generally attributed to chromatin decondensation. Loss of heterochromatin associated with ageing is well documented in yeast [22] and other model organisms [23] and in syndromes of premature ageing such as Hutchinson–Gilford progeria syndrome (HGPS) [24]. Furthermore, a process known as relocation of chromatin-modifying factors (RCM) [22] exemplified by the checkpoint-dependent repositioning of sirtuins (yeast Sir2/mammalian SIRT1) to sites of stress induces transcriptional deregulation that reflects an expression profile reminiscent of ageing [25, 26].

In replicative senescence, histone deacetylase (HDAC) activity diminishes [27, 28], corresponding histone acetylation increases, whereas there is a decline in global DNA methylation ([29] and references therein). Interestingly, treatment with HDAC inhibitors such as sodium butyrate or trichostatin A induces senescence [28, 30-32] arguing that chromatin relaxation plays a causative role in senescence. Although the reported chromatin changes occur globally, individual promoters are uniquely modified (e.g. cell cycle inhibitors [33, 34]) and only the expression of a subset of genes is altered in senescence [35] suggesting the existence of a targeted transcriptional program rather than global transcriptional alterations.

OPN is a multifunctional signaling molecule [36]. Originally identified in cancer cells [37], the physiological function of OPN is linked to matrix integrity and bone maintenance [38]. However, extensive research has implicated OPN in every stage of transformation and has led to its classification as a prognostic factor for several malignancies [39-43]. We have previously reported increased levels of secreted OPN in senescent fibroblasts and identified it as a critical mediator of stromal-epithelial interactions in tumorigenesis [9]. Given its diversity in signaling, it is not surprising that OPN is under the control of a variety of transcriptional factors that are cell type specific [44]. However, how OPN is regulated in senescent cells remains unknown.

Here we report that OPN upregulation in senescence is independent of the p53 and Rb pathways. Contrary to IL6 and IL8, whose concomitant overexpression is controlled by NfκB, OPN is insensitive to NfκB signaling. Furthermore, while we

observe a robust activation of the DNA damage response upon the induction of senescence, we found that inhibition of ATM had no impact on the activation of OPN. Importantly, treatment with an agent capable of directly perturbing chromatin structure in the absence of DNA breaks was an equally potent senescence inducer that led to OPN and SASP activation. I assessed the activity of several fragments of the human OPN promoter in young and senescent fibroblasts thus identifying a particular region that is the most responsive in senescence. Using the most responsive OPN promoter fragment, I conducted an RNAi-screen to identify putative transcription factors involved in the regulation of OPN in senescence. Together our results indicate that the senescent secretory phenotype although characterized as a collective transcriptional response, is under various layers of regulation with chromatin playing a central role.

Materials and methods

Cell lines and treatments. Human foreskin BJ fibroblasts and 293T cells were grown as previously described [9]. Human fetal lung IMR90 fibroblasts were grown in MEM media supplemented with 15% non-heat inactivated FBS (Sigma, St. Louis, MO). Human AT fibroblasts (GM09607) were purchased from Coriell Institute (Camden, New Jersey) and grown under the same conditions as IMR90 fibroblasts. Fibroblasts were mock or Bleomycin sulfate-treated (100ug/ml, Sigma, St. Louis, MO) for 24 hrs to induce senescence. Replicatively senescent fibroblasts were obtained by continuous passage.

After 72 hr serum-starvation, RNA was collected using TRIzol (Invitrogen, Carlsbad, CA). Actinomycin D (Sigma) was added to the cells after bleomycin treatment. For sodium butyrate (Sigma), cells were incubated with two fresh changes of 4mM for 72 hr.

Plasmids. The pBabe-IκBα-mut construct [45] was purchased from Addgene (Boston, MA plasmid 15291). The NfκB promoter luciferase construct, NF-κB_5-luc and pGL3-Renilla were a kind gift from David Piwnica-Worms (Washington University, St. Louis, MO). Two shRNA plasmids targeting the human ATM gene sequence were provided in the pLKO.1 plasmid (sequences found on Mission shRNA website; Sigma, St. Louis, MO). Human p27 cDNA was cloned in pBabe-puromycin. The pBabe-p53-DD and pBabe-DK constructs were provided by Robert Weinberg (Whitehead Institute, Cambridge, MA). Human OPN promoter constructs [46] were kindly provided by Dr. Paul Kuo (Duke University, Durham, NC)

Real-time PCR. Standard protocol was followed for cDNA and RT-PCR using the following primers (IDT, Coralville, IA):

OPN F - TTGCAGCCTTCTCAGCCAA; R 5'- AAGCAAATCACTGCAATTCTC,

GAPDH, F -GCATGGCCTTCCGTGTCC, R 5'-ATGCCAGCCCCAGCGTCAAA

IL6, F-ACATCCTCGACGGCATCTCA-3', R -TCACCAGGCAAGTCTCCTCA

IL8, F -GCTCTGTGTGAAGGTGCAGT-, R –TGCACCCAGTTTTCCTTGGG

SIRT6 F-AGGATGTCGGTGAATACGC R- AAAGGTGGTGTCGAACTTGG

H2A.X F- GGCCTTTCACATCAGCTCTC R- ATTGCCGAGTGAGTTTTGCT

TIP60 F- AACCAGGACAACGAAGATGAGT; R- GTGATCTGGACCGGGATTGG

ATM (Taqman, ABI, Carlsbad, CA cat.# Hs01112317-g1).

Luciferase Assay. Cells were co-transfected with pGL3-Renilla and NF-κB_5-luc or OPN promoter fragments using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Readings were performed with a Dual-Luciferase Reporter Assay (Promega, Madison, WI).

Western Blot Analysis. Fibroblast cell pellets were lysed in 100ul of RIPA buffer (50mM HEPES pH 7.4, 150mM NaCl, 1% Triton, 1M EDTA, 10% glycerol) and protein was quantified by the Bradford Protein Assay (Bio Rad, Hercules, CA). The following primary antibodies were used: p421 (a p53 hybridoma supernatant kindly provided by Robert Weinberg [Whitehead Institute, Cambridge, MA]) at 1:100; Cdk4 (Santa Cruz Biotechnologies, Santa Cruz, CA) at 1:200**;** β Actin (Sigma, St. Louis, MO) at 1:10,000**;** γ-actin (Sigma, St. Louis, MO) at 1:5000; γH2AX (Upstate, Billerica, MA) at 1:1000 and detected by the appropriate HRP-conjugated secondary antibodies (Sigma, St. Louis, MO).

Virus production and retroviral infections. Virus preparation as previously described [9].

Immunofluorescent staining. Fibroblasts were plated on coverslips, serum-starved overnight and fixed with 4% paraformaldehyde (Electronic Microscopy Services, Hatfield, PA). After permeabilization with 1% TritonX-100, the samples were stained with the following primary antibodies: γH2AX (Upstate, Billerica, MA) at 1:300 and

phospho-ATM (Cell Signaling, Danvers, MA cat.# 4526) at 1:500, for 1.5 hr at room temperature. Specific staining was detected by fluoro-conjugated secondary antibodies (Invitrogen, Carlsbad, CA).

Senescent-associated β-galactosidase. Staining was performed on cells as previously described [47].

Results

We previously performed microarray analysis on young, replicatively senescent (RS) and stress-induced prematurely senescent (SIPS) BJ fibroblasts and observed marked differences in their expression profiles [9]. This work led us to focus on understanding the role of one of the upregulated proteins in senescence – OPN, in the cross-talk between fibroblasts and epithelial cells. Indeed, we found that OPN was necessary to stimulate the *in vitro* and *in vivo* growth of preneoplastic keratinocytes. Given the significance of OPN in this model and the importance of the other senescent secretory proteins, we investigated how OPN expression was regulated in senescence. Treatment with bleomycin, a DNA damaging agent precipitates the onset of senescence and the associated transcriptional changes in a similar fashion to replicative senescence [9]. Both BJ and IMR90 fibroblasts experience a robust upregulation of OPN mRNA upon induction of senescence with bleomycin treatment (**Figure 3.1A**). To confirm that such upregulation reflects an increase in de novo transcription, we examined OPN levels in senescence in the presence of actinomycin D, a well-characterized transcription

inhibitor [48]. The classical OPN upregulation associated with senescence was significantly reduced in the presence of actinomycin D (**Figure 3.1B**) indicating that indeed OPN is regulated at the transcriptional level.

p53 and Rb are essential for the induction and maintenance of senescence via transcription of cell cycle inhibitors and repression of proliferation genes respectively [49]. Furthermore, OPN was identified as a direct transcriptional target of p53 [50]. To investigate whether p53 and Rb activity were required for OPN mRNA upregulation in senescent cells, we introduced a dominant negative p53 tetramerization domain (p53-DD) [51] and a cyclin dependent kinase 4^{R24C} (Cdk4)-cyclin D1 fusion protein (DK) [52] that effectively block activation of p53 and inactivation of Rb respectively (**Figure 3.2A**). Neither abrogation of p53 nor Rb reduced OPN transcript upon the activation of senescence. On the contrary, in the absence of p53 or Rb, OPN basal levels in young cells increased and they were augmented further upon induction of senescence. This suggested that p53 and Rb not only are irrelevant for the senescence-associated stimulation of OPN transcription but they in fact suppress it (**Figure 3.2B)**. As previously reported, IL6 and IL8 levels in senescence also increased when p53 and Rb function was compromised (**Supplemental Figure S3.1** and [2]), indicating that these factors are similarly affected by the p53 and Rb pathways in senescence.

NfκB is a master regulator of cytokine production in inflammatory responses [53] and recently it was invoked in the transcriptional control of SASP [4, 12]. Like IL6, OPN can participate in processes characterized by chronic inflammation [54] and NfκB

can directly activate its transcription under certain conditions [55]. Therefore, it was plausible that NfκB directly activated OPN in senescent cells. NfκB is classically trapped in the cytoplasm in a complex with IκBα, which upon stimulation is phosphorylated leading to degradation, thus liberating NfκB and allowing it to translocate to the nucleus and activate transcription of its target genes [56]. To evaluate the importance of NfκB in the transcriptional activation of OPN in senescence, I blocked its signaling in young and senescent cells by expressing a mutant of IκBα that cannot be phosphorylated (IκBα-mut) [45] (**Figure 3.2C**). In agreement with previous reports [4], we found that NfκB activation is essential for the upregulation of IL6 and IL8 in senescence (**Figure 3.2D**). In contrast, OPN levels remained unperturbed upon inhibition of NfκB (**Figure 3.2D**). Because the expression of the mutant precedes the induction of senescence, it indicates that NfκB signaling is not required for the initiation or the maintenance of OPN levels in senescence.

Having established that neither the p53 or Rb pathways nor activation of NfκB played a role in the upregulation of OPN in response to the activation of senescence, we next turned our attention to the putative role of DDR. Senescence is characterized by a robust and persistent DNA damage response [17] (**Figure 3.3A**) that includes activation of the ATM kinase [49] and ATM activation, which has previously been linked to the control of SASP [11]. To address whether ATM activation was required for the upregulation of OPN in senescent cells we utilized ATM specific short hairpin constructs to deplete ATM from our cells (**Figure 3.3B**). Despite a greater than 80 percent

reduction in ATM mRNA, we did not observe a reduction in OPN levels upon the induction of senescence (**Figure 3.3C**). In contrast, we found that the senescence-associated upregulation of IL6 and IL8 was significantly impacted upon the inhibition of ATM (**Figure 3.3C**) as previously reported [11]. These results indicate that OPN unlike IL6 and IL8 is not controlled by DDR and NfκB signaling and represents divergent regulation of the senescent secretory profile.

To confirm that the senescence-associated upregulation of OPN was independent of DNA breaks, we induced senescence via overexpression of p27, an inhibitor of cyclin-CDK complexes [57] or treatment with the histone deacetylase inhibitor sodium butyrate (NaB) [31, 32]. In comparison to cells transduced with an empty vector, cells expressing p27 cDNA assumed a flattened phenotype accompanied by positive staining for SA-βgal and growth arrest (**Figure 3.4A**). Similarly, treatment with NaB resulted in lack of cellular proliferation and a flattened phenotype consistent with senescence features (data not shown and [30]).

To confirm that treatment with NaB did not cause DNA breaks, we carried out a Comet assay, a sensitive technique utilized to detect DNA damage [58]. As expected, treatment with NaB did not result in DNA breaks (data not shown) [30]. To further corroborate these results, we also evaluated the levels of γH2AX, a phosphorylated form of histone H2AX, widely recognized as an early marker of DDR and a platform for assembly of repair factors [59]. Western blot analysis of an irradiated sample demonstrated a robust increase in γH2AX as expected; while, neither p27 expression nor

NaB treatment led to increased γH2AX levels in comparison to control cells (**Figure 3.4B**). Next, we examined OPN levels in cells driven to senescence by p27 expression or NaB treatment. In both settings, we observed a robust induction of OPN despite the lack of detectable DNA damage (**Figure 3.4C** and **D**). Notably, both IL6 and IL8 also underwent a drastic increase in cells ectopically expressing p27 or treated with NaB (**Figure 3.4C** and **D**) indicating that DNA breaks are not required for SASP induction.

Given that in bleomycin-induced senescence, ATM and NfκB are required for IL6 and IL8 regulation and NaB treatment activates ATM [81], we wondered whether the same regulatory mechanisms are employed in NaB-induced senescence. Therefore, we treated cells that express the IκBα-mut construct or AT cells (genetically deficient in ATM activity) with NaB. Although NaB does not induce physical DNA damage, we found that NfκB and ATM activity are mandatory for IL6 and IL8 expression; whereas in agreement with earlier they do not affect OPN upregulation in senescence (**Figure 3.5A** and **B** and data not shown). These results underscore the conserved nature of SASP activation in senescence and suggest that chromatin is a central modulator of these transcriptional pathways.

The human OPN promoter contains several binding sites for various transcription factors [60], which are differentially engaged depending on the cell type and signaling involved. The promoter elements required for its activation in senescence have not been identified. Therefore, I examined a set of fragments derived from the human OPN (hOPN) promoter [46] for their ability to respond in young and senescent fibroblasts.

Transient transfection of serial deletions of the hOPN promoter revealed a defined pattern of activation in senescence with a marked increase observed in the region above nucleotide -135 (**Fig. 3.6A**) suggesting that crucial elements reside there. Scanning this region for binding motifs yielded a repertoire of putative transcription factors (**Figure 3.6B**). We next investigated their role in OPN regulation by performing an unbiased RNAi-screen. The work described above indicated that chromatin modifications induce OPN and SASP activation. Therefore, I included in our RNAi screen both putative transcription factors for which binding sites were present in the senescent-responsive region of the OPN promoter and chromatin-modifying proteins previously implicated in senescence. Most of the transcription factors with putative binding sites on the promoter fragment (**Figure 3.6B**) were not detectable in senescent fibroblasts (data not shown); therefore, it is highly unlikely they are required for OPN expression in this setting. Hence, much of our effort has focused on chromatin-modifying enzymes. One exception was the transcription factor c-Myb. I found that the senescence-responsive OPN promoter fragment contains the sequence TAACTGT, which is an identical match to the Myb-binding site (MBS) [61] (**Figure 3.6B**). Indeed, c-Myb binds the OPN promoter *in vivo* in melanoma cells [62]. Appendix 1 contains preliminary studies suggesting a role for Myb involvement in the regulation of OPN in senescence.

To assess the potential role of chromatin-modifying enzymes in OPN regulation, I infected young BJ cells and induced senescence by blemoycin treatment. Surprisingly three independent hairpins that depleted TIP60, a histone acetyltransferase that is

required for ATM activation in response to DNA damage, by 50 percent had a dramatic effect on OPN expression in senescence [63-65] (**Figure 3.7A**). In addition to ATM, TIP60 targets several histones and is recruited to multiple promoters as a component of various chromatin-modifying complexes, which could account for the effect on OPN expression [65]. Additionally, I observed a significant decrease in OPN levels in senescent fibroblasts expressing hairpins targeting SIRT6 and H2A.X (**Figure 3.7B** and **C**). Two short hairpins that resulted in roughly 50 percent knockdown of SIRT6 at the mRNA level decreased OPN expression by more than 70 percent (**Figure 3.7B**). Sirtuins are well-characterized promoters of longevity and SIRT6 was shown to deacetylate H3K9 and attenuate NfκB signaling [66, 67]. Depletion of H2A.X, which like TIP60 participates in chromatin-mediated DNA damage sensing and repair [68, 69], by 80 percent by two independent hairpins, likewise affected OPN transcription. It is unclear in what capacity SIRT6 and H2A.X act in OPN activation; however, these results clearly indicate that disruption of the chromatin architecture impacts OPN and likely SASP activation in senescence.

Discussion

Senescent fibroblasts positively affect tumorigenesis in multiple models [6-8, 10, 70]. In efforts to understand how this is accomplished, several groups have examined the expression profile of senescent cells and have uncovered a signature secretory program enriched in growth factors, cytokines, and proteases termed senescence-associated secretory phenotype (SASP) [2, 4, 5, 9]. Specific components of SASP have been

directly implicated in senescent stromal-promoted tumorigenesis [5, 9, 10, 12]; however, it is still unclear as to how SASP is activated in senescence. Initial findings demonstrated that the transcription factors NfκB and C/EBPβ are essential for the upregulation of the inflammatory cytokines and indeed directly bind their promoters [4, 5, 12]. Furthermore, the DNA damage response (DDR) was invoked as an upstream inducer [11]. Indeed, the abovementioned transcription factors can be activated in response to DNA damage [71-73], although such relationship has not been described in senescence. Utilizing osteopontin (OPN) as a surrogate for SASP regulation, we found that SASP is not characterized by a single transcriptional axis but is instead a diverse program activated in response to chromatin changes.

OPN is significantly upregulated at the mRNA level in replicatively and stress-induced senescent fibroblasts [9]. While originally identified as a component of SASP by microarray analysis of early passage and senescent fibroblasts [9], sensitivity to actinomycin D confirmed that OPN upregulation was transcriptional (**Figure 3.1B**) as is the rest of SASP [2]. OPN is involved in a vast array of signaling pathways hence its transcription is governed by multiple mechanisms in a tissue and context-dependent manner [74]. We demonstrate that in senescence, OPN is not controlled by p53, Rb or NfκB although p53 and NfκB have binding sites on the OPN promoter and do bind it *in vivo* under certain conditions [50, 55]. The lack of p53 and Rb involvement is in agreement with previous work showing that these essential pathways for senescence are in fact dispensable for SASP activation [2] suggesting a decoupling of senescence

induction and transcriptional activation of paracrine factors. Our findings reveal yet another layer in SASP regulation. While NfκB and C/EBPβ directly activate IL6 and IL8 (**Figure 3.2** and data not shown), they are not required for OPN upregulation in senescence (**Figure 3.2** and data not shown). Similarly, ATM knockdown has a profound effect on IL6 and IL8 expression yet OPN increases to the same levels as in control cells (**Figure 3.3**). Together, these results indicate that IL6 and IL8 are activated by ATM and NfκB as it has been suggested [11, 12] and OPN is not.

Because OPN levels increase in senescence alongside IL6 and IL8, there must be a common inducer of SASP. Given that bleomycin causes double-stranded breaks, one can postulate that DNA damage triggers a transcriptional response and the ATM involvement would justify this assumption [75, 76]. However, it is clear that OPN is not regulated by the DDR, which begs the question whether DNA damage activates ATM-independent pathways or another upstream stimulus initiates separate signaling cascades. Our experiments with senescent fibroblasts obtained by sodium butyrate (NaB) treatment or ectopic expression of p27 demonstrate that in the absence of DNA breaks (and ensuing signaling as measured by H2A.X phosphorylation, see **Figure 3.4B**), SASP is still activated. Interestingly, ATM and NfκB are required for the upregulation of IL6 and IL8 (**Figure 3.5A** and **B**) in NaB-induced senescence as well, thus lending support to the argument that DNA damage per se is not required to activate SASP. Histone deacetylase inhibitors (HDACI) modify the chromatin mainly by inducing hyperacetylation of histone and nonhistone proteins [77, 78] resulting in sustained transcriptional changes.

Reportedly, HDAC activity decreases in replicative senescence exemplified by reduction in levels of particular HDACs [27, 28]. In fact, such a mechanism has been invoked in the chromatin changes observed in the prematurely senescent Hutchinson-Gilford progeria syndrome (HGPS) cells [24]. However, we did not observe noticeable differences in overall HDAC activity in senescent cells (data not shown). The data presented here allow us to present a model where ATM activation in bleomycin- and NaB-induced senescence occurs due to chromatin alteration (**Figure 3.7D**). It is known that DNA breaks induce changes in the surrounding chromatin [79], which facilitates signaling and repair; conversely, chromatin modifications trigger the DNA damage checkpoint [80]. Indeed, HDACI activate ATM [71, 81] and our data argues that the DDR required for SASP activation is not one emanating from breaks.

The interconnectedness between damage and chromatin easily explains the phenotypes of the TIP60 and H2A.X-depleted cells – two proteins known to modify the chromatin in the context of the DNA damage response [82, 83]. The question remains how do they impact OPN given that its expression in senescence is unaffected by ATM. Also, in NaB-treated cells there was no increase in γH2A.X arguing that this DNA damage-dependent modification is dispensable for SASP activation. Given that depletion of H2A.X results also in a decrease of IL6 and IL8 (data not shown); it argues for a general effect on transcription of H2A.X deposition in chromatin possibly invoking other modifications as well. TIP60 is part of the chromatin remodeling complex, NuA4, which acetylates histones and transcription factors [84]. Its yeast homolog, Esa1p, is

ubiquitously recruited to promoters throughout the yeast genome [85]. Given the highly conserved nature of NuA4, the mammalian TIP60 may also act as a general cofactor for basal transcription. Indeed, in mammalian cells TIP60 has been shown to couple its activity to several transcription factors [65]. In this capacity, TIP60 (and H2A.X) may be recruited to the OPN promoter during senescence and acetylate the histones or specific transcription factors required for activation. It is less clear what role SIRT6 plays in OPN expression. In mammalian cells SIRT6 deacetylates H3K9 [66]. Cells isolated from SIRT6-/- mice have an enhanced expression of NfkB targets and undergo premature senescence (SIRT6 null mice have a progeroid phenotype) [66, 67]. Under these circumstances, SIRT6 activity inhibits the expression of pro aging genes; whereas in our system, SIRT6 is necessary for OPN activation in senescence. Although, mouse and human cells differ in the molecular execution of senescence [86], SASP appears to be conserved between the two under physiological oxygen levels [87].

How p27 or other inducers of senescence alter the chromatin and lead to activation of SASP is currently unknown. p27 is a cell cycle inhibitor, whose action results in Rb hypophosphorylation [88, 89]. Rb impacts chromatin by recruiting remodeling complexes to promoters [90], yet we demonstrate that Rb function is not required for SASP activation (**Figure 3.2B** and **Supplemental Figure S3.1**). Therefore, it is more plausible that p27 acts as the initial trigger of cell cycle arrest but the chromatin modifications germane to senescence in fact modulate SASP. While it is unclear how chromatin changes are instituted and maintained in senescence, there is ample evidence

for their presence [18-21, 26, 91, 92]. It is conceivable that widespread chromatin modifications impact promoter activity globally; however, this seems unlikely given that our microarray results indicate the activation of only a subset of genes upon induction of senescence. Furthermore, specific transcription factors (e.g. NfκB and C/EBPβ) are required for the expression of a subset of SASP. Nevertheless, there is no single transcriptional axis. It is unlikely that there are as many transcription factors activated as the number of genes in SASP. Given that IL6, IL8 and several other inflammatory cytokines are controlled by NfκB and C/EBPβ [4, 5], it is reasonable to speculate that subsets of SASP have individual direct regulators. In fact, SASP contains different clusters as defined by GO term analysis [9]. Our results indicate that SASP is not the result of a single homogeneous transcriptional program and OPN represents a separate cluster from the inflammatory cytokines [4, 5] and it will be interesting to uncover activators of OPN and determine whether they control the expression of other proteins belonging to the extracellular matrix cluster [9]. How chromatin in senescence serves as a template for SASP activation and whether particular modifications are required is the fundamental question to address in the future.

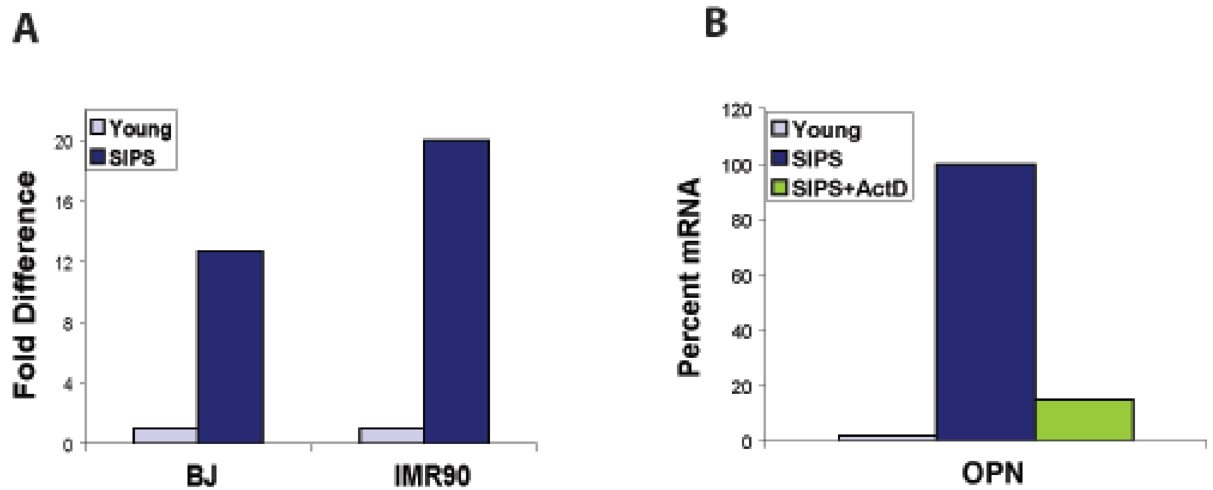


Figure 3.1 Osteopontin is transcriptionally upregulated in senescence. A, Stress-induced prematurely senescent (SIPS) BJ or IMR90 human fibroblasts obtained by bleomycin treatment were serum-starved for 72hr and analyzed for OPN mRNA levels by quantitative real-time PCR (qRT-PCR). OPN expression in young cells was set to 1. B, Effect of the transcriptional inhibitor actinomycin D was assessed on the senescence-associated OPN upregulation. OPN expression in SIPS was set to 100 percent (n=2).

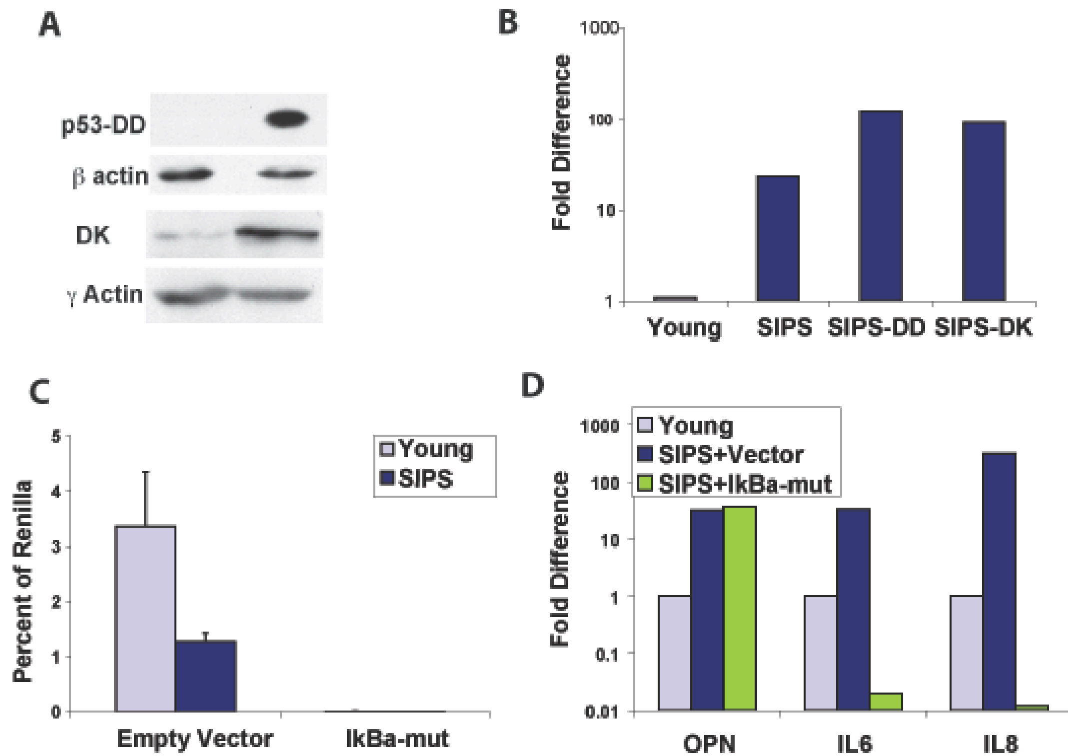


Figure 3.2 p53, Rb, and NfκB are dispensable for OPN regulation in senescent fibroblasts. A, Expression of a mutant form of p53 (p53-DD) or a cyclin D-cdk4 fusion (DK) proteins in BJ fibroblasts is shown by Western Blot analysis. B, OPN levels were measured by qRT-PCR in senescent BJ cells overexpressing p53-DD and DK. Expression in young BJ cells was set to 1 (n=2). C, Relative luciferase levels of an NfκB responsive promoter (pNF-κB_5-luc) were quantified in young and senescent fibroblasts expressing an empty vector or an IκBα super-repressor mutant (IκBα-mut) (n=2). D, Same cells as in C were assayed for mRNA levels of OPN, IL6 and IL8. Levels of expression in young cells was set to 1 (n=2).

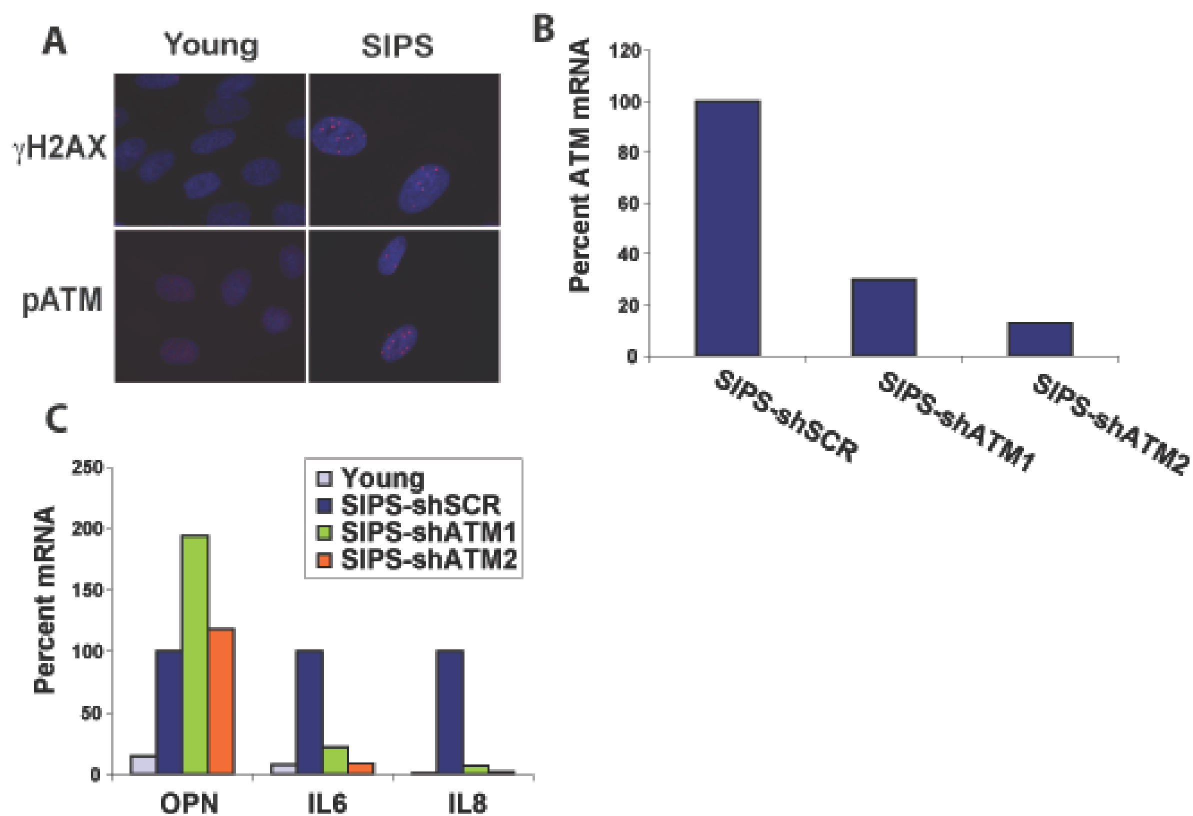


Figure 3.3 ATM regulates only a subset of SASP. A, Immunofluorescent images of young and senescent BJ fibroblasts stained for the presence of γH2AX and phosphorylated ATM (pATM) foci. B, ATM mRNA levels in BJ fibroblasts after the introduction of a control hairpin (shSCR) or specific hairpins targeting ATM. Expression in the control hairpin cells was set at 1 (n=2). C, Same cells as in B were assayed for the mRNA levels of OPN, IL6, and IL8. Expression in the control hairpin cells was set at 1 (n=2)

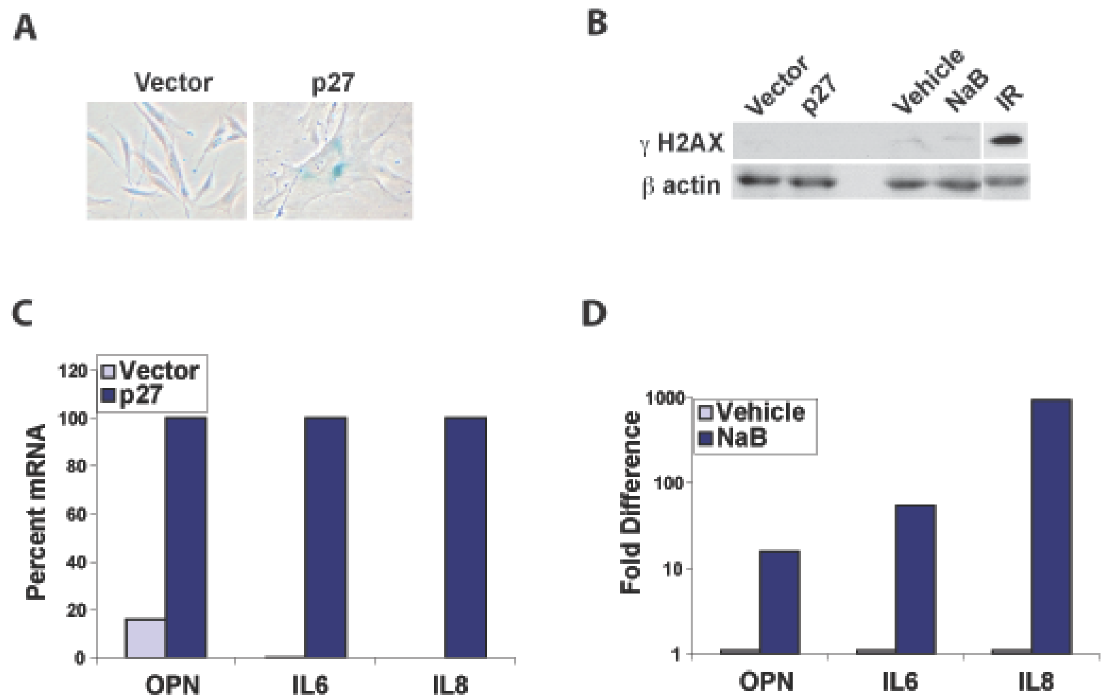


Figure 3.4 DNA breaks are not required for the upregulation of OPN in senescence. A, Senescence-associated β galactosidase (SA-βgal) staining of BJ fibroblasts that ectopically express p27. B, Immunoblot analysis of γH2AX levels in the cells described in A. A sample irradiated with 5 Gy is included as a control. C and D, mRNA levels of OPN, IL6 and IL8 in BJ fibroblasts that ectopically express p27 or treated with NaB respectively (n=3).

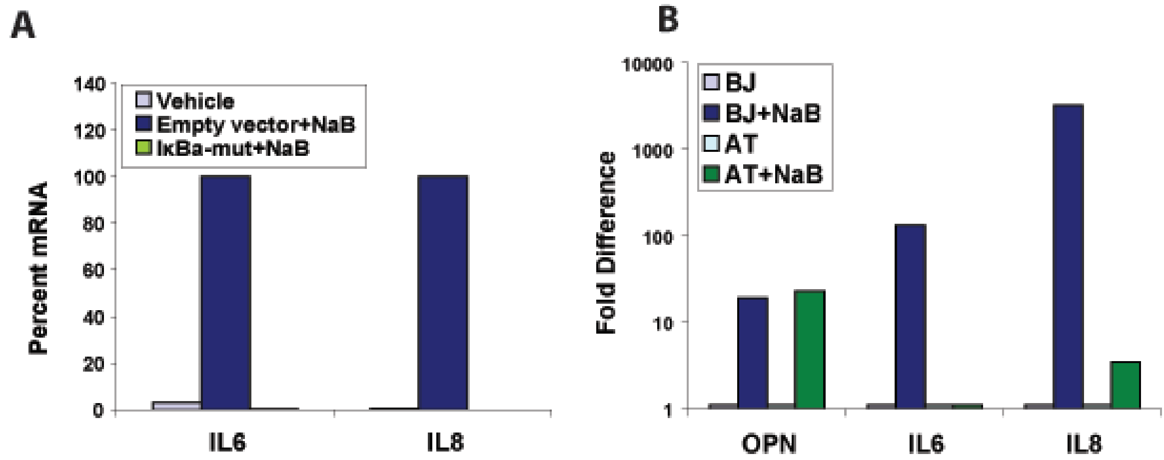
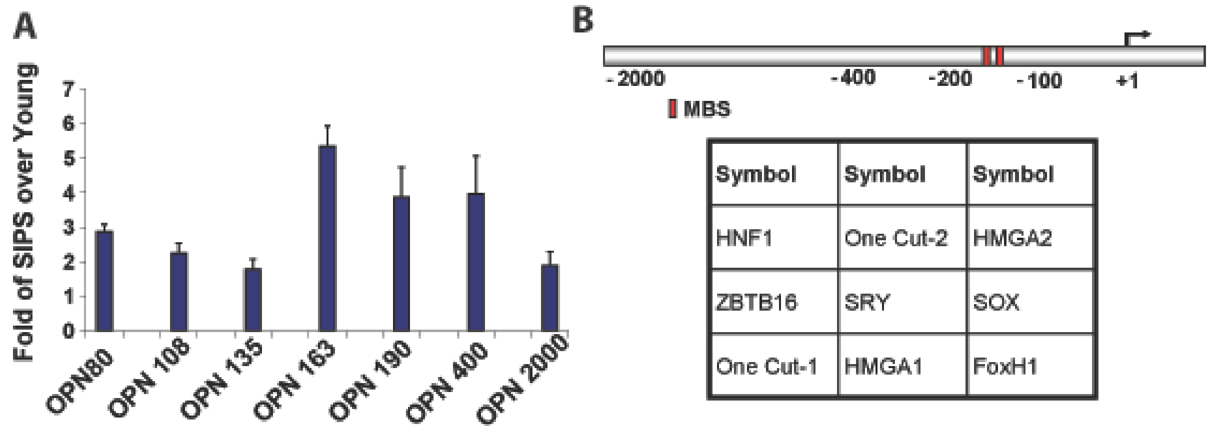


Figure 3.5 ATM and NfκB are required for IL6 an IL8 transcription in NaB-induced senescence. A, BJ fibroblasts overexpressing IκBα-mut were treated with NaB and IL6 and IL8 mRNA levels were examined by qRT-PCR (n=2). B, AT cells were treated with NaB and qRT-PCR was performed to examine mRNA levels of OPN, IL6, and IL8 (n=2).



Symbol	Symbol	Symbol
HNF1	One Cut-2	HMGA2
ZBTB16	SRY	SOX
One Cut-1	HMGA1	FoxH1

Figure 3.6 Activity of the OPN promoter in senescence. A, Young and senescent BJ fibroblasts were transiently transfected with progressively longer constructs of the human OPN promoter driving the expression of luciferase. B, Transfac® analysis of the OPN 163 fragment identified several putative sites in the OPN promoter (table). Putative Myb (MBS) binding sites are annotated on the OPN 2000 fragment.

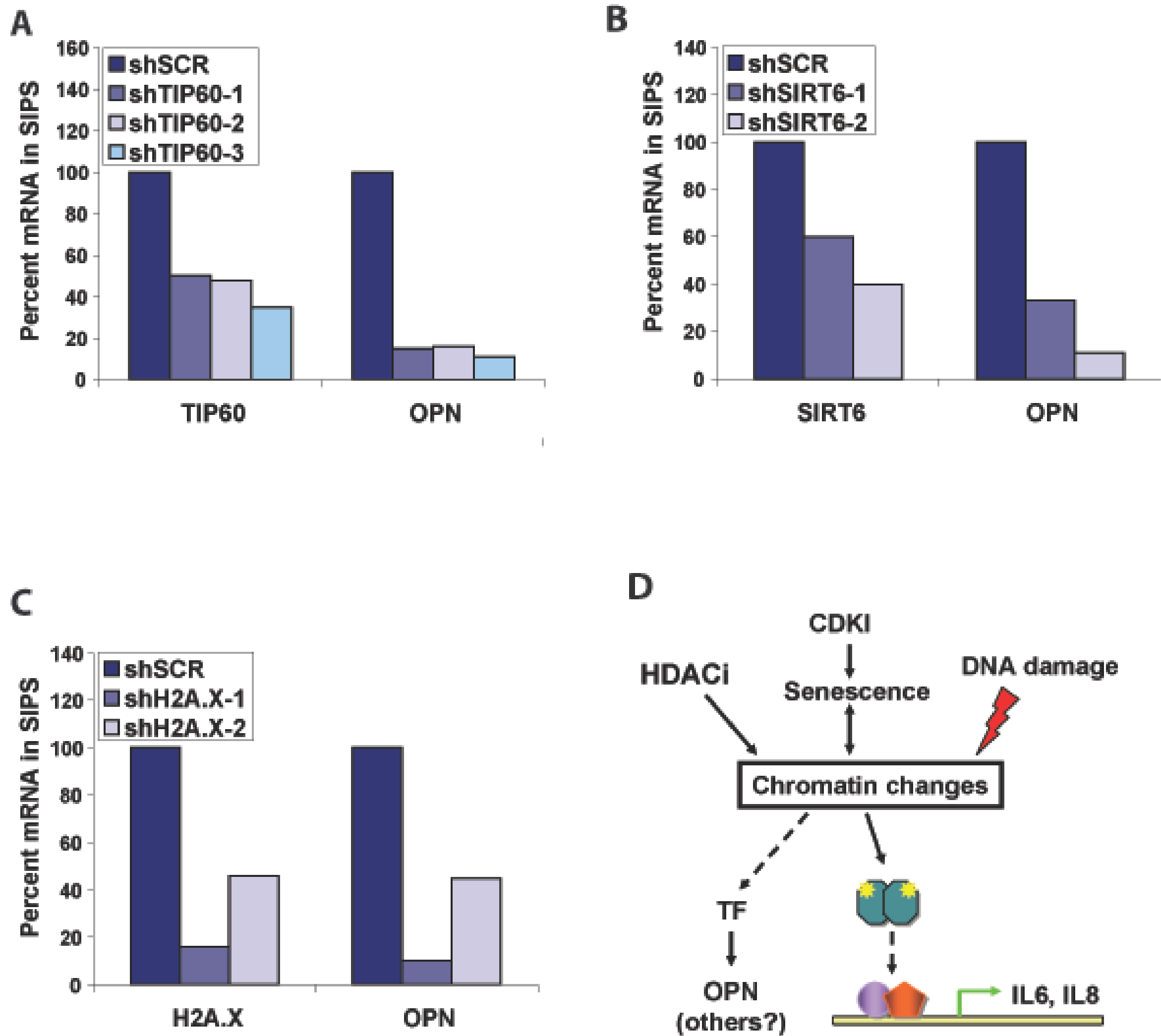
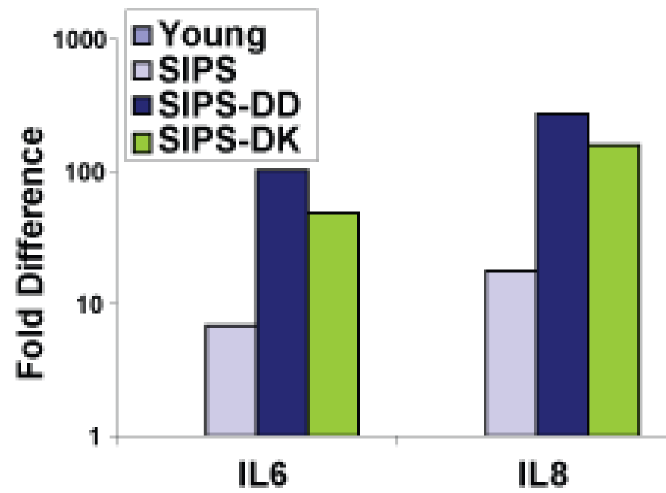


Figure 3.7 Chromatin modulators affect OPN expression. A, TIP60 mRNA (left) and OPN mRNA (right) in senescent BJ fibroblasts expressing short hairpins targeting TIP60. B, SIRT6 mRNA (left) and OPN mRNA (right) in senescent BJ fibroblasts expressing short hairpins targeting SIRT6. C, H2A.X mRNA (left) and OPN mRNA (right) in senescent BJ fibroblasts expressing short hairpins targeting H2A.X. D, Model of how chromatin alterations may lead to activation of SASP – modified chromatin through phosphorylated ATM (green) can activate NfκB, which directly binds the IL6 and IL8 promoters and induces the inflammatory subset of SASP. Other transcription factors (TF) can be activated by chromatin modifications and subsequently lead to OPN expression and possibly other members of SASP belonging to the same cluster.



Supplemental Figure S3.1 p53 and Rb are not required for IL6 and IL8 transcription. Fibroblasts expressing either a truncated form of p53 (DD) or cyclinD-cdk4 fusion construct (DK), which inhibits p53 and Rb respectively, were treated with bleomycin and mRNA levels of IL6 and IL8 were measured by qRT-PCR (n=2).

References

1. Pazolli, E., and Stewart, S.A. (2008). Senescence: the good the bad and the dysfunctional. Curr Opin Genet Dev *18*, 42-47.
2. Coppe, J.P., Patil, C.K., Rodier, F., Sun, Y., Munoz, D.P., Goldstein, J., Nelson, P.S., Desprez, P.Y., and Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol *6*, 2853-2868.
3. Campisi, J., and d'Adda di Fagagna, F. (2007). Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol *8*, 729-740.
4. Acosta, J.C., O'Loghlen, A., Banito, A., Guijarro, M.V., Augert, A., Raguz, S., Fumagalli, M., Da Costa, M., Brown, C., Popov, N., Takatsu, Y., Melamed, J., d'Adda di Fagagna, F., Bernard, D., Hernando, E., and Gil, J. (2008). Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell *133*, 1006-1018.
5. Kuilman, T., Michaloglou, C., Vredeveld, L.C., Douma, S., van Doorn, R., Desmet, C.J., Aarden, L.A., Mooi, W.J., and Peeper, D.S. (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell *133*, 1019-1031.
6. Krtolica, A., Parrinello, S., Lockett, S., Desprez, P.Y., and Campisi, J. (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci U S A *98*, 12072-12077.
7. Yang, G., Rosen, D.G., Zhang, Z., Bast, R.C., Jr., Mills, G.B., Colacino, J.A., Mercado-Uribe, I., and Liu, J. (2006). The chemokine growth-regulated oncogene 1 (Gro-1) links RAS signaling to the senescence of stromal fibroblasts and ovarian tumorigenesis. Proc Natl Acad Sci U S A *103*, 16472-16477.
8. Liu, D., and Hornsby, P.J. (2007). Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. Cancer Res *67*, 3117-3126.
9. Pazolli, E., Luo, X., Brehm, S., Carbery, K., Chung, J.J., Prior, J.L., Doherty, J., Demehri, S., Salavaggione, L., Piwnica-Worms, D., and Stewart, S.A. (2009). Senescent stromal-derived osteopontin promotes preneoplastic cell growth. Cancer Res *69*, 1230-1239.
10. Parrinello, S., Coppe, J.P., Krtolica, A., and Campisi, J. (2005). Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. J Cell Sci *118*, 485-496.
11. Rodier, F., Coppe, J.P., Patil, C.K., Hoeijmakers, W.A., Munoz, D.P., Raza, S.R., Freund, A., Campeau, E., Davalos, A.R., and Campisi, J. (2009). Persistent DNA

damage signalling triggers senescence-associated inflammatory cytokine secretion. Nat Cell Biol *11*, 973-979.

12. Orjalo, A.V., Bhaumik, D., Gengler, B.K., Scott, G.K., and Campisi, J. (2009). Cell surface-bound IL-1alpha is an upstream regulator of the senescence-associated IL-6/IL-8 cytokine network. Proc Natl Acad Sci U S A *106*, 17031-17036.

13. Krizhanovsky, V., Yon, M., Dickins, R.A., Hearn, S., Simon, J., Miething, C., Yee, H., Zender, L., and Lowe, S.W. (2008). Senescence of activated stellate cells limits liver fibrosis. Cell *134*, 657-667.

14. Adler, A.S., Sinha, S., Kawahara, T.L., Zhang, J.Y., Segal, E., and Chang, H.Y. (2007). Motif module map reveals enforcement of aging by continual NF-kappaB activity. Genes Dev *21*, 3244-3257.

15. Sebastian, T., Malik, R., Thomas, S., Sage, J., and Johnson, P.F. (2005). C/EBPbeta cooperates with RB:E2F to implement Ras(V12)-induced cellular senescence. Embo J *24*, 3301-3312.

16. Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L.V., Kolettas, E., Niforou, K., Zoumpourlis, V.C., Takaoka, M., Nakagawa, H., Tort, F., Fugger, K., Johansson, F., Sehested, M., Andersen, C.L., Dyrskjot, L., Orntoft, T., Lukas, J., Kittas, C., Helleday, T., Halazonetis, T.D., Bartek, J., and Gorgoulis, V.G. (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature *444*, 633-637.

17. Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P.G., Bensimon, A., Maestro, R., Pelicci, P.G., and d'Adda di Fagagna, F. (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638-642.

18. Narita, M., Nunez, S., Heard, E., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., and Lowe, S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell *113*, 703-716.

19. Zhang, R., Poustovoitov, M.V., Ye, X., Santos, H.A., Chen, W., Daganzo, S.M., Erzberger, J.P., Serebriiskii, I.G., Canutescu, A.A., Dunbrack, R.L., Pehrson, J.R., Berger, J.M., Kaufman, P.D., and Adams, P.D. (2005). Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Dev Cell *8*, 19-30.

20. Ye, X., Zerlanko, B., Zhang, R., Somaiah, N., Lipinski, M., Salomoni, P., and Adams, P.D. (2007). Definition of pRB- and p53-dependent and -independent steps in HIRA/ASF1a-mediated formation of senescence-associated heterochromatin foci. Mol Cell Biol *27*, 2452-2465.

21. Banumathy, G., Somaiah, N., Zhang, R., Tang, Y., Hoffmann, J., Andrake, M., Ceulemans, H., Schultz, D., Marmorstein, R., and Adams, P.D. (2009). Human UBN1 is an ortholog of yeast Hpc2p and has an essential role in the HIRA/ASF1a chromatin-remodeling pathway in senescent cells. Mol Cell Biol *29*, 758-770.

22. Oberdoerffer, P., and Sinclair, D.A. (2007). The role of nuclear architecture in genomic instability and ageing. Nat Rev Mol Cell Biol *8*, 692-702.

23. Haithcock, E., Dayani, Y., Neufeld, E., Zahand, A.J., Feinstein, N., Mattout, A., Gruenbaum, Y., and Liu, J. (2005). Age-related changes of nuclear architecture in Caenorhabditis elegans. Proc Natl Acad Sci U S A *102*, 16690-16695.

24. Pegoraro, G., Kubben, N., Wickert, U., Gohler, H., Hoffmann, K., and Misteli, T. (2009). Ageing-related chromatin defects through loss of the NURD complex. Nat Cell Biol *11*, 1261-1267.

25. Martin, S.G., Laroche, T., Suka, N., Grunstein, M., and Gasser, S.M. (1999). Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast. Cell *97*, 621-633.

26. Oberdoerffer, P., Michan, S., McVay, M., Mostoslavsky, R., Vann, J., Park, S.K., Hartlerode, A., Stegmuller, J., Hafner, A., Loerch, P., Wright, S.M., Mills, K.D., Bonni, A., Yankner, B.A., Scully, R., Prolla, T.A., Alt, F.W., and Sinclair, D.A. (2008). SIRT1 redistribution on chromatin promotes genomic stability but alters gene expression during aging. Cell *135*, 907-918.

27. Wagner, M., Brosch, G., Zwerschke, W., Seto, E., Loidl, P., and Jansen-Durr, P. (2001). Histone deacetylases in replicative senescence: evidence for a senescence-specific form of HDAC-2. FEBS Lett *499*, 101-106.

28. Place, R.F., Noonan, E.J., and Giardina, C. (2005). HDACs and the senescent phenotype of WI-38 cells. BMC Cell Biol *6*, 37.

29. Calvanese, V., Lara, E., Kahn, A., and Fraga, M.F. (2009). The role of epigenetics in aging and age-related diseases. Ageing Res Rev *8*, 268-276.

30. Pospelova, T.V., Demidenko, Z.N., Bukreeva, E.I., Pospelov, V.A., Gudkov, A.V., and Blagosklonny, M.V. (2009). Pseudo-DNA damage response in senescent cells. Cell Cycle *8*, 4112-4118.

31. Munro, J., Barr, N.I., Ireland, H., Morrison, V., and Parkinson, E.K. (2004). Histone deacetylase inhibitors induce a senescence-like state in human cells by a p16-dependent mechanism that is independent of a mitotic clock. Exp Cell Res *295*, 525-538.

32. Ogryzko, V.V., Hirai, T.H., Russanova, V.R., Barbie, D.A., and Howard, B.H. (1996). Human fibroblast commitment to a senescence-like state in response to histone deacetylase inhibitors is cell cycle dependent. Mol Cell Biol *16*, 5210-5218.

33. Kim, W.Y., and Sharpless, N.E. (2006). The regulation of INK4/ARF in cancer and aging. Cell *127*, 265-275.

34. Gil, J., and Peters, G. (2006). Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nat Rev Mol Cell Biol *7*, 667-677.

35. Shelton, D.N., Chang, E., Whittier, P.S., Choi, D., and Funk, W.D. (1999). Microarray analysis of replicative senescence. Curr Biol *9*, 939-945.

36. Rangaswami, H., Bulbule, A., and Kundu, G.C. (2006). Osteopontin: role in cell signaling and cancer progression. Trends Cell Biol *16*, 79-87.

37. Senger, D.R., Wirth, D.F., and Hynes, R.O. (1979). Transformed mammalian cells secrete specific proteins and phosphoproteins. Cell *16*, 885-893.

38. Rittling, S.R., and Denhardt, D.T. (1999). Osteopontin function in pathology: lessons from osteopontin-deficient mice. Exp Nephrol *7*, 103-113.

39. Koopmann, J., Fedarko, N.S., Jain, A., Maitra, A., Iacobuzio-Donahue, C., Rahman, A., Hruban, R.H., Yeo, C.J., and Goggins, M. (2004). Evaluation of osteopontin as biomarker for pancreatic adenocarcinoma. Cancer Epidemiol Biomarkers Prev *13*, 487-491.

40. Fedarko, N.S., Jain, A., Karadag, A., Van Eman, M.R., and Fisher, L.W. (2001). Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. Clin Cancer Res *7*, 4060-4066.

41. Bramwell, V.H., Doig, G.S., Tuck, A.B., Wilson, S.M., Tonkin, K.S., Tomiak, A., Perera, F., Vandenberg, T.A., and Chambers, A.F. (2006). Serial plasma osteopontin levels have prognostic value in metastatic breast cancer. Clin Cancer Res *12*, 3337-3343.

42. Wang, X., Chao, L., Ma, G., Chen, L., Tian, B., Zang, Y., and Sun, J. (2008). Increased expression of osteopontin in patients with triple-negative breast cancer. Eur J Clin Invest *38*, 438-446.

43. Weber, G.F. (2001). The metastasis gene osteopontin: a candidate target for cancer therapy. Biochim Biophys Acta *1552*, 61-85.

44. El-Tanani, M., Platt-Higgins, A., Rudland, P.S., and Campbell, F.C. (2004). Ets gene PEA3 cooperates with beta-catenin-Lef-1 and c-Jun in regulation of osteopontin transcription. J Biol Chem *279*, 20794-20806.

45. Boehm, J.S., Zhao, J.J., Yao, J., Kim, S.Y., Firestein, R., Dunn, I.F., Sjostrom, S.K., Garraway, L.A., Weremowicz, S., Richardson, A.L., Greulich, H., Stewart, C.J., Mulvey, L.A., Shen, R.R., Ambrogio, L., Hirozane-Kishikawa, T., Hill, D.E., Vidal, M., Meyerson, M., Grenier, J.K., Hinkle, G., Root, D.E., Roberts, T.M., Lander, E.S., Polyak, K., and Hahn, W.C. (2007). Integrative genomic approaches identify IKBKE as a breast cancer oncogene. Cell *129*, 1065-1079.

46. Takami, Y., Russell, M.B., Gao, C., Mi, Z., Guo, H., Mantyh, C.R., and Kuo, P.C. (2007). Sp1 regulates osteopontin expression in SW480 human colon adenocarcinoma cells. Surgery *142*, 163-169.

47. Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., and et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A *92*, 9363-9367.

48. Sobell, H.M. (1985). Actinomycin and DNA transcription. Proc Natl Acad Sci U S A *82*, 5328-5331.

49. d'Adda di Fagagna, F. (2008). Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer *8*, 512-522.

50. Morimoto, I., Sasaki, Y., Ishida, S., Imai, K., and Tokino, T. (2002). Identification of the osteopontin gene as a direct target of TP53. Genes Chromosomes Cancer *33*, 270-278.

51. Rheinwald, J.G., Hahn, W.C., Ramsey, M.R., Wu, J.Y., Guo, Z., Tsao, H., De Luca, M., Catricala, C., and O'Toole, K.M. (2002). A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. Mol Cell Biol *22*, 5157-5172.

52. Herbig, U., Wei, W., Dutriaux, A., Jobling, W.A., and Sedivy, J.M. (2003). Real-time imaging of transcriptional activation in live cells reveals rapid up-regulation of the cyclin-dependent kinase inhibitor gene CDKN1A in replicative cellular senescence. Aging Cell *2*, 295-304.

53. Karin, M. (2009). NF-kappaB as a critical link between inflammation and cancer. Cold Spring Harb Perspect Biol *1*, a000141.

54. Cho, H.J., and Kim, H.S. (2009). Osteopontin: a multifunctional protein at the crossroads of inflammation, atherosclerosis, and vascular calcification. Curr Atheroscler Rep *11*, 206-213.

55. Renault, M.A., Jalvy, S., Potier, M., Belloc, I., Genot, E., Dekker, L.V., Desgranges, C., and Gadeau, A.P. (2005). UTP induces osteopontin expression through a coordinate action of NFkappaB, activator protein-1, and upstream stimulatory factor in arterial smooth muscle cells. J Biol Chem *280*, 2708-2713.

56. Karin, M., and Greten, F.R. (2005). NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol *5*, 749-759.

57. Alexander, K., and Hinds, P.W. (2001). Requirement for p27(KIP1) in retinoblastoma protein-mediated senescence. Mol Cell Biol *21*, 3616-3631.

58. Singh, N.P., McCoy, M.T., Tice, R.R., and Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res *175*, 184-191.

59. Paull, T.T., Rogakou, E.P., Yamazaki, V., Kirchgessner, C.U., Gellert, M., and Bonner, W.M. (2000). A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol *10*, 886-895.

60. Hijiya, N., Setoguchi, M., Matsuura, K., Higuchi, Y., Akizuki, S., and Yamamoto, S. (1994). Cloning and characterization of the human osteopontin gene and its promoter. Biochem J *303 (Pt 1)*, 255-262.

61. Ness, S.A., Marknell, A., and Graf, T. (1989). The v-myb oncogene product binds to and activates the promyelocyte-specific mim-1 gene. Cell *59*, 1115-1125.

62. Schultz, J., Lorenz, P., Ibrahim, S.M., Kundt, G., Gross, G., and Kunz, M. (2009). The functional -443T/C osteopontin promoter polymorphism influences osteopontin gene expression in melanoma cells via binding of c-Myb transcription factor. Mol Carcinog *48*, 14-23.

63. Ikura, T., Ogryzko, V.V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J., and Nakatani, Y. (2000). Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell *102*, 463-473.

64. Sun, Y., Jiang, X., Chen, S., Fernandes, N., and Price, B.D. (2005). A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. Proc Natl Acad Sci U S A *102*, 13182-13187.

65. Squatrito, M., Gorrini, C., and Amati, B. (2006). Tip60 in DNA damage response and growth control: many tricks in one HAT. Trends Cell Biol *16*, 433-442.

66. Michishita, E., McCord, R.A., Berber, E., Kioi, M., Padilla-Nash, H., Damian, M., Cheung, P., Kusumoto, R., Kawahara, T.L., Barrett, J.C., Chang, H.Y., Bohr, V.A., Ried, T., Gozani, O., and Chua, K.F. (2008). SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. Nature *452*, 492-496.

67. Kawahara, T.L., Michishita, E., Adler, A.S., Damian, M., Berber, E., Lin, M., McCord, R.A., Ongaigui, K.C., Boxer, L.D., Chang, H.Y., and Chua, K.F. (2009). SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. Cell *136*, 62-74.

68. Riches, L.C., Lynch, A.M., and Gooderham, N.J. (2008). Early events in the mammalian response to DNA double-strand breaks. Mutagenesis *23*, 331-339.

69. Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K., and Bonner, W. (2002). Histone H2A variants H2AX and H2AZ. Curr Opin Genet Dev *12*, 162-169.

70. Bavik, C., Coleman, I., Dean, J.P., Knudsen, B., Plymate, S., and Nelson, P.S. (2006). The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. Cancer Res *66*, 794-802.

71. Wuerzberger-Davis, S.M., Nakamura, Y., Seufzer, B.J., and Miyamoto, S. (2007). NF-kappaB activation by combinations of NEMO SUMOylation and ATM activation stresses in the absence of DNA damage. Oncogene *26*, 641-651.

72. Wu, Z.H., Shi, Y., Tibbetts, R.S., and Miyamoto, S. (2006). Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli. Science *311*, 1141-1146.

73. Elkon, R., Rashi-Elkeles, S., Lerenthal, Y., Linhart, C., Tenne, T., Amariglio, N., Rechavi, G., Shamir, R., and Shiloh, Y. (2005). Dissection of a DNA-damage-induced transcriptional network using a combination of microarrays, RNA interference and computational promoter analysis. Genome Biol *6*, R43.

74. Chakraborty, G., Jain, S., Behera, R., Ahmed, M., Sharma, P., Kumar, V., and Kundu, G.C. (2006). The multifaceted roles of osteopontin in cell signaling, tumor progression and angiogenesis. Curr Mol Med *6*, 819-830.

75. Bredemeyer, A.L., Helmink, B.A., Innes, C.L., Calderon, B., McGinnis, L.M., Mahowald, G.K., Gapud, E.J., Walker, L.M., Collins, J.B., Weaver, B.K., Mandik-Nayak, L., Schreiber, R.D., Allen, P.M., May, M.J., Paules, R.S., Bassing, C.H., and Sleckman, B.P. (2008). DNA double-strand breaks activate a multi-functional genetic program in developing lymphocytes. Nature *456*, 819-823.

76. Gasser, S., Orsulic, S., Brown, E.J., and Raulet, D.H. (2005). The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature *436*, 1186-1190.

77. Candido, E.P., Reeves, R., and Davie, J.R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. Cell *14*, 105-113.

78. Buchwald, M., Kramer, O.H., and Heinzel, T. (2009). HDACi--targets beyond chromatin. Cancer Lett *280*, 160-167.

79. Kruhlak, M.J., Celeste, A., Dellaire, G., Fernandez-Capetillo, O., Muller, W.G., McNally, J.G., Bazett-Jones, D.P., and Nussenzweig, A. (2006). Changes in chromatin structure and mobility in living cells at sites of DNA double-strand breaks. J Cell Biol *172*, 823-834.

80. van Attikum, H., and Gasser, S.M. (2009). Crosstalk between histone modifications during the DNA damage response. Trends Cell Biol *19*, 207-217.

81. Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature *421*, 499-506.

82. Sedelnikova, O.A., Pilch, D.R., Redon, C., and Bonner, W.M. (2003). Histone H2AX in DNA damage and repair. Cancer Biol Ther *2*, 233-235.

83. Sun, Y., Jiang, X., and Price, B.D. Tip60: connecting chromatin to DNA damage signaling. Cell Cycle *9*, 930-936.

84. Doyon, Y., Selleck, W., Lane, W.S., Tan, S., and Cote, J. (2004). Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans. Mol Cell Biol *24*, 1884-1896.

85. Robert, F., Pokholok, D.K., Hannett, N.M., Rinaldi, N.J., Chandy, M., Rolfe, A., Workman, J.L., Gifford, D.K., and Young, R.A. (2004). Global position and recruitment of HATs and HDACs in the yeast genome. Mol Cell *16*, 199-209.

86. Itahana, K., Campisi, J., and Dimri, G.P. (2004). Mechanisms of cellular senescence in human and mouse cells. Biogerontology *5*, 1-10.

87. Coppe, J.P., Patil, C.K., Rodier, F., Krtolica, A., Beausejour, C.M., Parrinello, S., Hodgson, J.G., Chin, K., Desprez, P.Y., and Campisi, J. A human-like senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen. PLoS One *5*, e9188.

88. Toyoshima, H., and Hunter, T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell *78*, 67-74.

89. Sherr, C.J., and McCormick, F. (2002). The RB and p53 pathways in cancer. Cancer Cell *2*, 103-112.

90. Ferreira, R., Naguibneva, I., Pritchard, L.L., Ait-Si-Ali, S., and Harel-Bellan, A. (2001). The Rb/chromatin connection and epigenetic control: opinion. Oncogene *20*, 3128-3133.

91. Narita, M., Krizhanovsky, V., Nunez, S., Chicas, A., Hearn, S.A., Myers, M.P., and Lowe, S.W. (2006). A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. Cell *126*, 503-514.

92. Narita, M. (2007). Cellular senescence and chromatin organisation. Br J Cancer *96*, 686-691.

Chapter 4: Conclusions and Future Directions

4.1 Summary

Our understanding of tumors as complex organs has increased our appreciation for each component of the tumor microenvironment and its respective contribution to tumorigenesis. Fibroblasts found within a tumor actively participate in the growth, progression and metastasis of cancer cells. Senescent fibroblasts, which are permanently arrested yet metabolically active, accumulate in tissue over time where they may promote the proliferation and malignant conversion of preneoplastic cells. In chapter 2, I investigate the relationship between senescent fibroblasts and preneoplastic keratinocytes and identify the secreted multifunctional protein osteopontin (OPN) as a critical stromal mediating factor. RNAi-directed reduction of stromal OPN leads to decreased growth of preneoplastic keratinocytes *in vitro* and *in vivo.* Furthermore, I demonstrate the presence of senescent stroma and associated OPN expression in the early stages of a chemical carcinogenesis mouse model. OPN is part of a unique transcriptional profile activated upon senescence induction. Given the significance of senescent stromal-derived factors in tumorigenesis, I address the regulation of OPN and other members of the senescence-associated secretory profile (SASP) in chapter 3. Although SASP is coordinately upregulated in response to multiple stimuli inducing senescence, the downstream mechanisms responsible for the transcriptional activation of particular subsets of SASP are different. Work in chapter 3 illustrates the divergent regulation of OPN. I demonstrate that unlike the best-characterized SASP factors IL6 and IL8, OPN is insensitive to NfκB inhibition. Additionally, I examine the promoter of OPN and uncover a putative role for chromatin modulators in the regulation of OPN expression in

senescence. Below, I discuss the implications of my thesis work in the context of senescence and tumorigenesis at large. Additionally, I deliberate on the outstanding questions and how future studies may address them.

4.2 OPN as a Critical Senescent Stromal Factor

Osteopontin was identified several decades ago as a protein associated with transformed cells [1]. Most of the subsequent work focused on its role in tumorigenesis, where it has been implicated in various stages of transformation including survival, proliferation, angiogenesis, and metastasis [2]. Numerous studies qualify OPN as a prognostic candidate for several malignancies [3-7]. While OPN performs important functions in bone maintenance, matrix mineralization, and calcification; the impact of its loss is subtle as evidenced by the absence of any overt phenotypes in the OPN null mouse [8, 9], suggesting the existence of compensatory mechanisms under normal physiological settings. OPN's role in pathological settings appears to be more significant. Indeed, OPN baseline levels remain relatively low in most tissues unless there is a pathological challenge, which typically causes an immediate and significant spike in OPN expression thus classifying OPN as a general early stress responder [10]. Therefore, it is not surprising that when OPN null mice are challenged by various stresses, they display notable defects [9].

This is best illustrated by tumor studies. When OPN null mice are treated with a two-step chemical carcinogenesis protocol, they have a delayed onset and lower incidence of papillomas [11]. One explanation for this finding may be that the initiated

keratinocytes require OPN for early clonal expansion and growth – a prerequisite for papilloma formation. In fact, activation of H-Ras, which is the mutational target in this model [12], leads to OPN transcription [13, 14]. However, data discussed in chapter 2 argues that additional properties of OPN may account for the delayed tumor phenotype in the OPN null mice. I found that senescent fibroblasts, unlike their genetically identical younger counterparts, express high levels of OPN. Examination of papillomas revealed the presence of senescent stroma that expressed OPN (**Figure 2.3**). Importantly, I observed senescent stroma in the skin of treated mice long before the emergence of papillomas arguing that senescent stromal-derived OPN plays a role in the initial stages of tumorigenesis. These findings could explain the delayed onset and lower incidence of papillomas in the OPN null mouse given that we also showed that stromal-derived OPN is necessary for the growth of premalignant keratinocytes (**Figure 2.4**).

Recently, it was shown that OPN is a member of a pro-inflammatory cancer-associated fibroblast (CAF) signature, which is responsible for recruiting macrophages and promoting angiogenesis and tumor progression in a skin model of tumorigenesis [15]. This report supports our observations of reduced immune infiltrate in xenografts with fibroblasts expressing a short hairpin against OPN (**Figure 2.5**). Additionally, it demonstrates that CAFs are similar to senescent stroma in that they are found in dysplastic lesions again arguing that fibroblasts are an important contributor early on in the transformation process. Given the importance of the stromal compartment in tumorigenesis, these results raise the possibility that manipulation of the stroma before any neoplastic changes occur may impact tumor formation.

To this end, a conditional mouse model would be better suited to address the role of OPN derived from different sources. To investigate the function of senescent stroma in tumorigenesis, the laboratory has undertaken the creation of two different mouse strains engineered to induce senescence exclusively in the stromal compartment via ectopic expression of p27 (already shown to induce senescence in fibroblasts *in vitro* in Chapter 3 and discussed below) or telomeric repeat factor 2 (TRF2) deletion, which will be subjected to various tumorigenesis protocols including the two-step chemical carcinogenesis described above. TRF2 is a component of the critical telomeric protective complex known as shelterin [16] – once deleted leads to rapid telomere dysfunction and cellular senescence [17]. Once these models have been fully validated, they can be utilized to assess the role of OPN in the context of senescent stromal promotion of tumorigenesis. While mating into the OPN null mouse does not exclude contribution from other cell types, senescence will be induced conditionally in the stroma and a comparison with the matched control will uncover any differences derived from the absence of OPN in the senescent stromal compartment. Alternatively, a conditional deletion of OPN in the fibroblasts would be the ideal model of study. All of the above experimental paradigms are complex and time demanding yet important to parse out the effects of OPN in tumorigenesis.

Our data indicate that OPN acts locally in a paracrine fashion by stimulating the growth of preneoplastic keratinocytes; yet it has systemic effects reflected in the recruitment of immune cells. This has also been demonstrated with tumor-derived OPN, which attracts bone marrow-derived cells into indolent tumors thereby fueling their

growth [18]. It is unclear whether senescent stromal-derived OPN accomplishes the same task. The xenograft model is not robust enough to address this issue given that the fate of the injected senescent fibroblasts is uncertain. However, the aforementioned conditional models may provide a better platform for this purpose. There is more impetus to study and target stromal-derived OPN given its significant local and systemic impact.

OPN can affect multiple parameters of tumorigenesis and this depends on the cell type and modifications present [19]. I found that senescent fibroblasts promote the proliferation of preneoplastic keratinocytes *in vitro* as evidenced by higher BrdU incorporation and unaffected apoptotic rates in the presence of senescent fibroblasts compared to young fibroblasts (data not shown). Ongoing work in the laboratory has confirmed this finding when examining the effect of OPN alone on keratinocytes' growth and further uncovering a putative role for the MAPK pathway as the downstream mediator of OPN's proliferative signaling. The main caveat of this approach is the utilization of human recombinant OPN. Although OPN is extensively modified, it is unknown whether these modifications recapitulate those found in the OPN produced by senescent fibroblasts. OPN is a 34 kDa protein, yet it typically runs at a higher molecular weight due to abundant phosphorylation and glycosylation [20], which is the case with the senescence-derived OPN (**Figure S2.3**). Isolation and purification of OPN derived from senescent fibroblasts would provide an ideal tool to study the signaling initiated by senescent fibroblasts in their cross-talk with preneoplastic keratinocytes.

4.3 Induction and Regulation of SASP

What controls the decision of a cell to enter senescence remains largely unknown; however, some mechanistic insights have emerged in the recent years. Several reports demonstrated that eroded telomeres, which trigger replicative senescence, are recognized as sites of DNA damage and therefore elicit a persistent DNA damage response (DDR) [21-23]. DDR initiation activates the downstream transcription factor p53, which implements cellular arrest [24]. Remarkably, oncogene-induced senescence employs DDR as well [25, 26] revealing a common underlying mechanism between replicative and stress-induced senescence. In addition to p53, Rb represses proliferation by recruiting the heterochromatin machinery to E2F promoters [27]. Indeed, senescence in some cell types is accompanied by senescent-associated heterochromatic foci or SAHFs [27, 28]. Both a persistent DDR and marked chromatin modulation appear to be required for senescence [25, 29] although knowledge of explicit mechanisms is lacking.

In addition to permanent proliferative arrest, senescent cells are characterized by an altered transcriptional profile [30], which is referred to as the senescence-associated secretory profile or SASP [31] because it includes several chemokines, growth factors, and enzymes that are secretory in nature and capable of altering the surrounding microenvironment. Regulation of SASP is the subject of intensive research, which has focused on whether the same mechanisms that govern cell cycle arrest also direct the SASP transcriptional program. I investigated this question by examining OPN transcriptional regulation in senescence. Unexpectedly, neither p53 nor Rb activity were required for OPN expression in senescence (**Figure 3.2B**). On the contrary, OPN levels

were augmented further upon the induction of senescence in the absence of p53 or Rb, following the same trend as other SASP components including the well characterized IL6 and IL8 [31]. Since SASP promotes tumorigenesis in a paracrine fashion, it has been argued that p53 acts as a tumor suppressor in a non cell-autonomous fashion in addition to its classic cell-autonomous role [31].

Although p53 is one of the major targets of the DNA damage response (DDR) [32], it is not required for SASP transcription [31]. However, DDR activates multiple pathways in addition to p53 [33]. Furthermore, there is precedence for DDR activating transcription of genes that are not involved in the downstream repair process [34, 35]. Therefore, I assessed the impact of DDR on OPN expression in senescence and found that ATM inhibition did not affect OPN levels (**Figure 3.3)**. Conversely, expression of IL6 and IL8 was dramatically reduced in agreement with previous results [36]. This was one indication that OPN transcription is subject to different regulation and that SASP consists of distinct transcriptional clusters. Another element that set OPN regulation apart was the insensitivity to NfκB activity, which was crucial for IL6 and IL8 expression (**Figure 3**.**2**). Therefore, senescence-associated OPN transcription, unlike IL6 and IL8 does not require the actions of ATM or NfκB.

To confirm the general applicability of these findings, I induced senescence by different methods that unlike bleomycin treatment do not induce DNA breaks. When cells are exposed to sodium butyrate (NaB) a histone deacetylae inhibitor (HDACI) or when they ectopically express the cyclin inhibitor p27, they become senescent. Given that OPN is a SASP factor that does not require DNA damage for its activation, we

expected a similar upregulation in p27- and NaB-induced senescence to that detected in bleomycin-induced senescence. Indeed, I did observe that OPN transcription was increased following both of these treatments (**Figure 3.4C** and **D**). Strikingly, IL6 and IL8 also underwent a significant upregulation in both settings indicating that they are not strictly induced by DNA breaks. However, we found that IL6 and IL8 transcription failed to increase when ATM or NfκB were abrogated in NaB-treated cells, arguing that these pathways are still critical in the absence of DNA damage (**Figure 3.5**). Mechanistically, DNA breaks induce sumoylation of NEMO (a modulator of classic NfκB signaling), which is recognized by ATM and further modified by phosphorylation, removal of SUMO, and ubiquitination. The resultant complex is exported out of the nucleus and subsequently binds the IKK complex thus allowing the release of NfκB and its translocation into the nucleus [37]. It is conceivable that NfκB activation in bleomycin-induced senescence, which incurs damage occurs through this pathway. One can examine the interaction between ATM and NEMO and determine the effect of the ATM-phospho mutant NEMO [37] on IL6 and IL8 expression in senescence. On the other hand, HDACI activation of NfκB is attributed to continuous acetylation of p65, which reduces the affinity of NfκB for its negative regulatory IκB complex and ensures continuous signaling [38] and ATM may be involved in some cells [39]. This is testable by utilizing acetylation mutants of p65 and rescuing with HDAC3 (the HDAC responsible for deacetylation of NfκB).

These experiments begin to address the fundamental question of what signaling nexus is active in senescence and how it triggers SASP. DDR and chromatin modulation

are inextricably linked. DNA breaks cause remodeling of the surrounding chromatin [40]; however, the transient changes in chromatin architecture that accommodate DNA damage sensing and repair cannot be responsible for SASP given that transient DNA damage is not sufficient for SASP induction [36]. Conversely, chromatin modulation causes activation of DNA damage kinases [41]. Moreover, it is important to note that DNA damage signaling (e.g. activation of DDR kinases such as ATR) in the absence of breaks can induce senescence [42]. In our experiments with NaB we failed to detect DNA breaks by the sensitive COMET assay (data not shown and [43]). Furthermore, we did not detect any increases in γH2AX levels upon NaB treatment arguing against any evidence of ongoing DNA damage signaling (**Figure 3.4B**). Significantly, we do not observe phosphorylated ATM aggregated in foci in NaB-treated cells; instead there is a diffuse pattern indicative of activation in the absence of breaks ([41] and data not shown). One can surmise from the above observations and the requirement of ATM for IL6 and IL8 transcription in NaB-treated cells (**Figure 3.5**) that ATM is activated in a break-independent manner.

Addressing how chromatin remodeling triggers SASP activation is a much more difficult task due to the unknown nature of modifications occurring in senescence. HDACI induce histone hyperacetylation that leads to changes in gene expression patterns [44-46]. Interestingly, in replicative senescence HDAC1 protein levels are lower [47, 48]. Similarly, HDAC1 levels and corresponding activity are also lower in cells obtained from patients with premature ageing syndromes such as Hutchinson-Gilford progeria syndrome (HGPS), which are characterized by gross defects in nuclear architecture and

chromatin [49]. Reduction of histone deacetylase activity and silencing of specific heterchromatic complexes (NuRD) alone recapitulates the nuclear defects of HGPS cells arguing that chromatin modifications playing a causative role in premature ageing [49]. Significantly, gene expression changes including upregulation of extracellular matrix (ECM) genes occur in HGPS cells [50]. We have observed a modest OPN upregulation in HGPS cells (data not shown), which prompted me to investigate whether a similar mechanism operates in senescence. However to date I have not detected any significant changes in HDAC1 levels or overall HDAC activity in bleomycin-induced senescence (data not shown) suggesting that other components of the chromatin machinery may be involved.

Work done in model organisms provides an alternative hypothesis to transcriptional alterations in ageing [51]. Sirtuins, which are NAD-dependent HDAC, are well known promoters of longevity [52]. During genotoxic stress, sirtuins relocate from their telomeric positions to sites of damage, where they contribute to the assembly of additional chromatin factors. Once repair is accomplished, sirtuins return to their usual location. This phenomenon is known as relocation of chromatin modifiers or RCM [53]. However, during normal ageing or chronic genotoxic stress, the spatial flexibility of RCM decreases and sirtuins are stably relocated to promoters of genes typically upregulated during ageing [53]. One of the transcription factors modulated by SIRT6 is NfκB [54], which controls several age-associated genes [55] and at least a subset of the SASP [56, 57]. Although the level of sirtuins does not change, their ability to respond adequately to stress and maintain telomere stability is decreased presumably resulting in

ageing phenotypes [51]. Notably, short hairpins that target SIRT6 significantly decrease OPN levels in senescence (**Figure 3.7**). While classically sirtuins are implicated in facultative heterochromatin formation and repression of transcription [53, 58], their effects on gene expression can be variable depending on the target promoter. For example, SIRT1-mediated deacetylation of FOXO3 favors activation of genes mediating cell arrest but not apoptosis [59]. Thus, sirtuins could be occupying specific promoters themselves as part of a chromatin complex and/or modifying the activity of transcription factor(s) required for activation [59-63]. Additionally, SIRT1 deacetylates SUV39H1 increasing its activity [58], thereby possibly affecting senescence induction [29] and indirectly SASP activation. Chromatin-immunoprecipiation for specific sirtuins or examining interactions with putative transcription factors could clarify this issue.

How senescence induced by p27 overexpression activates SASP is unclear. Chromatin modulation in this context has not been investigated. p27 arrests the cell cycle by inhibiting cyclin/Cdk complexes ultimately leading to Rb activation [64]. Rb is a well-characterized mediator of chromatin remodeling by virtue of its association with several modifying complexes [65]. As previously mentioned, it is required for SAHF formation in senescence [27]. However, experiments performed with cells expressing the DK mutant [66] (**Figure 3.2**) negate any Rb involvement (the DK fusion protein should block the activation of all pocket proteins including p107 and p130 [67]). p27 has other non-cell cycle dependent functions and modifications by various kinases coordinate its actions mainly outside the nucleus [68]. Paradoxically, p27 thus modified is usually associated with proliferation and tumor progression [68]. Given that cell cycle arrest and

SASP induction appear to be independently regulated [31], it is possible that noncanonical targets of p27 yet to be elucidated play a role in SASP activation.

Several lines of evidence suggest that independent of the inducer of senescence, unifying mechanisms are in place to activate SASP. Global histone and specific promoter analysis would be useful to determine whether particular chromatin modifications correlate with SASP activation. Probing for various overall modifications including methylation and acetylation of histones could lead to the identification of potential key modulators of SASP. For instance, histone methyltransferase SUV39H1, which trimethylates H3K9 is required for the induction of senescence [29]. H3K9me3 is a repressive mark that mediates gene silencing in senescence including E2F-controlled cell cycle regulators [27]. Acetyltransferases perform the opposite function and thereby may activate SASP. Depletion of TIP60 – a HAT found in several chromatin modifying complexes [69], decreases OPN levels in senescence (**Figure 3.7**). It will be interesting to examine whether its HAT activity is required for OPN expression (upcoming experiments will test more thoroughly the effect of a TIP60 construct mutated in its HAT function) [70]. While promoter analysis of SASP factors is expected to confirm an open chromatin configuration in senescence, particular marks may lead to the elucidation of a specific modulator/complex, which can be validated by chromatin immunoprecipitation.

Whether particular chromatin modulations dictate transcription factor activation in senescence is currently unknown. Our preliminary results invoke c-Myb as putative transcriptional regulator of OPN in senescence since its depletion led to a decline in OPN expression (**Figure A1.3**). c-Myb has a binding site on the OPN promoter (**Figure 3.6B**).

Ongoing experiments will validate these results and determine whether c-Myb binds *in vivo* upon induction of senescence by utilizing chromatin immunoprecipitation.

Independently of the result of the above experiment, the question remains as to how these transcription factors are activated in senescence. There is no change in the mRNA or protein levels of c-Myb (**Figure A1.4** and data not shown) suggesting that posttranslational modifications or conditional partnering is responsible. c-Myb is subject to phosphorylation, sumoylation, ubiquitination, and acetylation [71], which can affect its binding and/or transactivational activity. Probing for any of these modifications may provide clues to its regulation in senescence. We have ruled out the involvement of TGFβ in the activation of OPN transcription (**Appendix 2**); instead our results implicate the MAPK pathways particularly Mek (**Figure A2.5**) in the activation of OPN expression upon induction of senescence. We need to confirm preliminary results demonstrating ERK activation in senescence and corroborate the effect of MEK inhibition on OPN regulation by genetic mutants (e.g. MEK DN). Interestingly, c-Myb is a target of ERK [72] suggesting an upstream regulator. Finally, we have demonstrated that OPN is not under the same transcriptional control as IL6 and IL8, which begs the question whether OPN belongs to a different SASP cluster. IL6 and IL8 represent the inflammatory component of SASP [31, 56, 57] and it is not surprising that NfκB, a master inflammatory orchestrator [73], regulates their expression even in senescence. Conversely, OPN is a matrix-associated protein and extracellular matrix proteins are significantly represented in the SASP [74, 75]. We have undertaken some preliminary analysis to identify additional SASP factors that are regulated similarly to OPN. A large

microarray study comparing young fibroblasts to senescent fibroblasts that express hairpins targeting c-Myb or other transcription factors involved will address this issue in a broader fashion and reveal any putative clusters.

4.4 Conclusions

The tumor microenvironment is a critical player in tumorigenesis. This work has focused on the role senescent fibroblasts play in tumor initiation and progression. We have identified osteopontin as a secreted protein enriched in senescent fibroblasts and have demonstrated that it is required for the paracrine stimulation of preneoplastic cells by senescent fibroblasts both in *in vitro* models and in xenograft studies. Such findings are revealing given that we have identified senescent stroma in the early steps in carcinogenesis *in vivo* and OPN expression by such stroma in early lesions such as skin papillomas [74]. Continuing efforts are aimed at understanding how the senescent stroma triggers the production of OPN and other SASP factors. We have excluded involvement of well-characterized transcriptional pathways employed in senescence and have demonstrated that SASP factors, while coordinately upregulated are subject to different transcriptional activators. While we continue to investigate how OPN and other senescent stromal factors are regulated, we will be able to understand better the complex heterotypic relationships characteristic of tumors. Our work may expose at the molecular level the link between increased cancer risk and ageing.

References

1. Senger, D.R., Wirth, D.F., and Hynes, R.O. (1979). Transformed mammalian cells secrete specific proteins and phosphoproteins. Cell *16*, 885-893.
2. Chakraborty, G., Jain, S., Behera, R., Ahmed, M., Sharma, P., Kumar, V., and Kundu, G.C. (2006). The multifaceted roles of osteopontin in cell signaling, tumor progression and angiogenesis. Curr Mol Med *6*, 819-830.
3. Fedarko, N.S., Jain, A., Karadag, A., Van Eman, M.R., and Fisher, L.W. (2001). Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. Clin Cancer Res *7*, 4060-4066.
4. Bramwell, V.H., Doig, G.S., Tuck, A.B., Wilson, S.M., Tonkin, K.S., Tomiak, A., Perera, F., Vandenberg, T.A., and Chambers, A.F. (2006). Serial plasma osteopontin levels have prognostic value in metastatic breast cancer. Clin Cancer Res *12*, 3337-3343.
5. Johnston, N.I., Gunasekharan, V.K., Ravindranath, A., O'Connell, C., Johnston, P.G., and El-Tanani, M.K. (2008). Osteopontin as a target for cancer therapy. Front Biosci *13*, 4361-4372.
6. Koopmann, J., Fedarko, N.S., Jain, A., Maitra, A., Iacobuzio-Donahue, C., Rahman, A., Hruban, R.H., Yeo, C.J., and Goggins, M. (2004). Evaluation of osteopontin as biomarker for pancreatic adenocarcinoma. Cancer Epidemiol Biomarkers Prev *13*, 487-491.
7. Wang, X., Chao, L., Ma, G., Chen, L., Tian, B., Zang, Y., and Sun, J. (2008). Increased expression of osteopontin in patients with triple-negative breast cancer. Eur J Clin Invest *38*, 438-446.
8. Liaw, L., Birk, D.E., Ballas, C.B., Whitsitt, J.S., Davidson, J.M., and Hogan, B.L. (1998). Altered wound healing in mice lacking a functional osteopontin gene (spp1). J Clin Invest *101*, 1468-1478.
9. Rittling, S.R., and Denhardt, D.T. (1999). Osteopontin function in pathology: lessons from osteopontin-deficient mice. Exp Nephrol *7*, 103-113.
10. Mazzali, M., Kipari, T., Ophascharoensuk, V., Wesson, J.A., Johnson, R., and Hughes, J. (2002). Osteopontin--a molecule for all seasons. Qjm *95*, 3-13.
11. Chang, P.L., Cao, M., and Hicks, P. (2003). Osteopontin induction is required for tumor promoter-induced transformation of preneoplastic mouse cells. Carcinogenesis *24*, 1749-1758.
12. Quintanilla, M., Brown, K., Ramsden, M., and Balmain, A. (1986). Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature *322*, 78-80.

13. Guo, X., Zhang, Y.P., Mitchell, D.A., Denhardt, D.T., and Chambers, A.F. (1995). Identification of a ras-activated enhancer in the mouse osteopontin promoter and its interaction with a putative ETS-related transcription factor whose activity correlates with the metastatic potential of the cell. Mol Cell Biol *15*, 476-487.

14. Wu, Y., Denhardt, D.T., and Rittling, S.R. (2000). Osteopontin is required for full expression of the transformed phenotype by the ras oncogene. Br J Cancer *83*, 156-163.

15. Erez, N., Truitt, M., Olson, P., Arron, S.T., and Hanahan, D. Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. Cancer Cell *17*, 135-147.

16. de Lange, T. (2002). Protection of mammalian telomeres. Oncogene *21*, 532-540.

17. de Lange, T. (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev *19*, 2100-2110.

18. McAllister, S.S., Gifford, A.M., Greiner, A.L., Kelleher, S.P., Saelzler, M.P., Ince, T.A., Reinhardt, F., Harris, L.N., Hylander, B.L., Repasky, E.A., and Weinberg, R.A. (2008). Systemic endocrine instigation of indolent tumor growth requires osteopontin. Cell *133*, 994-1005.

19. Christensen, B., Kazanecki, C.C., Petersen, T.E., Rittling, S.R., Denhardt, D.T., and Sorensen, E.S. (2007). Cell type-specific post-translational modifications of mouse osteopontin are associated with different adhesive properties. J Biol Chem *282*, 19463-19472.

20. Bayless, K.J., Davis, G.E., and Meininger, G.A. (1997). Isolation and biological properties of osteopontin from bovine milk. Protein Expr Purif *9*, 309-314.

21. Feldser, D.M., and Greider, C.W. (2007). Short telomeres limit tumor progression in vivo by inducing senescence. Cancer Cell *11*, 461-469.

22. Meier, A., Fiegler, H., Munoz, P., Ellis, P., Rigler, D., Langford, C., Blasco, M.A., Carter, N., and Jackson, S.P. (2007). Spreading of mammalian DNA-damage response factors studied by ChIP-chip at damaged telomeres. Embo J *26*, 2707-2718.

23. Reaper, P.M., di Fagagna, F., and Jackson, S.P. (2004). Activation of the DNA damage response by telomere attrition: a passage to cellular senescence. Cell Cycle *3*, 543-546.

24. d'Adda di Fagagna, F. (2008). Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer *8*, 512-522.

25. Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P.G., Bensimon, A., Maestro, R., Pelicci, P.G.,

and d'Adda di Fagagna, F. (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638-642.

26. Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L.V., Kolettas, E., Niforou, K., Zoumpourlis, V.C., Takaoka, M., Nakagawa, H., Tort, F., Fugger, K., Johansson, F., Sehested, M., Andersen, C.L., Dyrskjot, L., Orntoft, T., Lukas, J., Kittas, C., Helleday, T., Halazonetis, T.D., Bartek, J., and Gorgoulis, V.G. (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature *444*, 633-637.

27. Narita, M., Nunez, S., Heard, E., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., and Lowe, S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell *113*, 703-716.

28. Zhang, R., Poustovoitov, M.V., Ye, X., Santos, H.A., Chen, W., Daganzo, S.M., Erzberger, J.P., Serebriiskii, I.G., Canutescu, A.A., Dunbrack, R.L., Pehrson, J.R., Berger, J.M., Kaufman, P.D., and Adams, P.D. (2005). Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Dev Cell *8*, 19-30.

29. Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A.H., Schlegelberger, B., Stein, H., Dorken, B., Jenuwein, T., and Schmitt, C.A. (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. Nature *436*, 660-665.

30. Shelton, D.N., Chang, E., Whittier, P.S., Choi, D., and Funk, W.D. (1999). Microarray analysis of replicative senescence. Curr Biol *9*, 939-945.

31. Coppe, J.P., Patil, C.K., Rodier, F., Sun, Y., Munoz, D.P., Goldstein, J., Nelson, P.S., Desprez, P.Y., and Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol *6*, 2853-2868.

32. Liu, Y., and Kulesz-Martin, M. (2001). p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding. Carcinogenesis *22*, 851-860.

33. Matsuoka, S., Ballif, B.A., Smogorzewska, A., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Bakalarski, C.E., Zhao, Z., Solimini, N., Lerenthal, Y., Shiloh, Y., Gygi, S.P., and Elledge, S.J. (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science *316*, 1160-1166.

34. Bredemeyer, A.L., Huang, C.Y., Walker, L.M., Bassing, C.H., and Sleckman, B.P. (2008). Aberrant V(D)J recombination in ataxia telangiectasia mutated-deficient lymphocytes is dependent on nonhomologous DNA end joining. J Immunol *181*, 2620-2625.

35. Gasser, S., Orsulic, S., Brown, E.J., and Raulet, D.H. (2005). The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature *436*, 1186-1190.

36. Rodier, F., Coppe, J.P., Patil, C.K., Hoeijmakers, W.A., Munoz, D.P., Raza, S.R., Freund, A., Campeau, E., Davalos, A.R., and Campisi, J. (2009). Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. Nat Cell Biol *11*, 973-979.

37. Wu, Z.H., Shi, Y., Tibbetts, R.S., and Miyamoto, S. (2006). Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli. Science *311*, 1141-1146.

38. Chen, L., Fischle, W., Verdin, E., and Greene, W.C. (2001). Duration of nuclear NF-kappaB action regulated by reversible acetylation. Science *293*, 1653-1657.

39. Rosato, R.R., Kolla, S.S., Hock, S.K., Almenara, J.A., Patel, A., Amin, S., Atadja, P., Fisher, P.B., Dent, P., and Grant, S. Histone deacetylase inhibitors activate NF-kappaB in human leukemia cells through an ATM/NEMO-related pathway. J Biol Chem *285*, 10064-10077.

40. Kruhlak, M.J., Celeste, A., Dellaire, G., Fernandez-Capetillo, O., Muller, W.G., McNally, J.G., Bazett-Jones, D.P., and Nussenzweig, A. (2006). Changes in chromatin structure and mobility in living cells at sites of DNA double-strand breaks. J Cell Biol *172*, 823-834.

41. Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature *421*, 499-506.

42. Toledo, L.I., Murga, M., Gutierrez-Martinez, P., Soria, R., and Fernandez-Capetillo, O. (2008). ATR signaling can drive cells into senescence in the absence of DNA breaks. Genes Dev *22*, 297-302.

43. Pospelova, T.V., Demidenko, Z.N., Bukreeva, E.I., Pospelov, V.A., Gudkov, A.V., and Blagosklonny, M.V. (2009). Pseudo-DNA damage response in senescent cells. Cell Cycle *8*, 4112-4118.

44. Van Lint, C., Emiliani, S., and Verdin, E. (1996). The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Expr *5*, 245-253.

45. Sambucetti, L.C., Fischer, D.D., Zabludoff, S., Kwon, P.O., Chamberlin, H., Trogani, N., Xu, H., and Cohen, D. (1999). Histone deacetylase inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects. J Biol Chem *274*, 34940-34947.

46. Davie, J.R. (2003). Inhibition of histone deacetylase activity by butyrate. J Nutr *133*, 2485S-2493S.

47. Wagner, M., Brosch, G., Zwerschke, W., Seto, E., Loidl, P., and Jansen-Durr, P. (2001). Histone deacetylases in replicative senescence: evidence for a senescence-specific form of HDAC-2. FEBS Lett *499*, 101-106.

48. Place, R.F., Noonan, E.J., and Giardina, C. (2005). HDACs and the senescent phenotype of WI-38 cells. BMC Cell Biol *6*, 37.

49. Pegoraro, G., Kubben, N., Wickert, U., Gohler, H., Hoffmann, K., and Misteli, T. (2009). Ageing-related chromatin defects through loss of the NURD complex. Nat Cell Biol *11*, 1261-1267.

50. Csoka, A.B., English, S.B., Simkevich, C.P., Ginzinger, D.G., Butte, A.J., Schatten, G.P., Rothman, F.G., and Sedivy, J.M. (2004). Genome-scale expression profiling of Hutchinson-Gilford progeria syndrome reveals widespread transcriptional misregulation leading to mesodermal/mesenchymal defects and accelerated atherosclerosis. Aging Cell *3*, 235-243.

51. Oberdoerffer, P., and Sinclair, D.A. (2007). The role of nuclear architecture in genomic instability and ageing. Nat Rev Mol Cell Biol *8*, 692-702.

52. Haigis, M.C., and Sinclair, D.A. Mammalian sirtuins: biological insights and disease relevance. Annu Rev Pathol *5*, 253-295.

53. Oberdoerffer, P., Michan, S., McVay, M., Mostoslavsky, R., Vann, J., Park, S.K., Hartlerode, A., Stegmuller, J., Hafner, A., Loerch, P., Wright, S.M., Mills, K.D., Bonni, A., Yankner, B.A., Scully, R., Prolla, T.A., Alt, F.W., and Sinclair, D.A. (2008). SIRT1 redistribution on chromatin promotes genomic stability but alters gene expression during aging. Cell *135*, 907-918.

54. Kawahara, T.L., Michishita, E., Adler, A.S., Damian, M., Berber, E., Lin, M., McCord, R.A., Ongaigui, K.C., Boxer, L.D., Chang, H.Y., and Chua, K.F. (2009). SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. Cell *136*, 62-74.

55. Adler, A.S., Sinha, S., Kawahara, T.L., Zhang, J.Y., Segal, E., and Chang, H.Y. (2007). Motif module map reveals enforcement of aging by continual NF-kappaB activity. Genes Dev *21*, 3244-3257.

56. Acosta, J.C., O'Loghlen, A., Banito, A., Guijarro, M.V., Augert, A., Raguz, S., Fumagalli, M., Da Costa, M., Brown, C., Popov, N., Takatsu, Y., Melamed, J., d'Adda di Fagagna, F., Bernard, D., Hernando, E., and Gil, J. (2008). Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell *133*, 1006-1018.

57. Kuilman, T., Michaloglou, C., Vredeveld, L.C., Douma, S., van Doorn, R., Desmet, C.J., Aarden, L.A., Mooi, W.J., and Peeper, D.S. (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell *133*, 1019-1031.

58. Vaquero, A., Scher, M., Erdjument-Bromage, H., Tempst, P., Serrano, L., and Reinberg, D. (2007). SIRT1 regulates the histone methyl-transferase SUV39H1 during heterochromatin formation. Nature *450*, 440-444.

59. Brunet, A., Sweeney, L.B., Sturgill, J.F., Chua, K.F., Greer, P.L., Lin, Y., Tran, H., Ross, S.E., Mostoslavsky, R., Cohen, H.Y., Hu, L.S., Cheng, H.L., Jedrychowski, M.P., Gygi, S.P., Sinclair, D.A., Alt, F.W., and Greenberg, M.E. (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-2015.

60. Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell *107*, 137-148.

61. Muth, V., Nadaud, S., Grummt, I., and Voit, R. (2001). Acetylation of TAF(I)68, a subunit of TIF-IB/SL1, activates RNA polymerase I transcription. Embo J *20*, 1353-1362.

62. Fulco, M., Schiltz, R.L., Iezzi, S., King, M.T., Zhao, P., Kashiwaya, Y., Hoffman, E., Veech, R.L., and Sartorelli, V. (2003). Sir2 regulates skeletal muscle differentiation as a potential sensor of the redox state. Mol Cell *12*, 51-62.

63. Yeung, F., Hoberg, J.E., Ramsey, C.S., Keller, M.D., Jones, D.R., Frye, R.A., and Mayo, M.W. (2004). Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. Embo J *23*, 2369-2380.

64. Sheaff, R.J. (1997). Regulation of mammalian cyclin-dependent kinase 2. Methods Enzymol *283*, 173-193.

65. Gonzalo, S., and Blasco, M.A. (2005). Role of Rb family in the epigenetic definition of chromatin. Cell Cycle *4*, 752-755.

66. Wei, W., Jobling, W.A., Chen, W., Hahn, W.C., and Sedivy, J.M. (2003). Abolition of cyclin-dependent kinase inhibitor p16Ink4a and p21Cip1/Waf1 functions permits Ras-induced anchorage-independent growth in telomerase-immortalized human fibroblasts. Mol Cell Biol *23*, 2859-2870.

67. Leng, X., Noble, M., Adams, P.D., Qin, J., and Harper, J.W. (2002). Reversal of growth suppression by p107 via direct phosphorylation by cyclin D1/cyclin-dependent kinase 4. Mol Cell Biol *22*, 2242-2254.

68. Lee, J., and Kim, S.S. (2009). The function of p27 KIP1 during tumor development. Exp Mol Med *41*, 765-771.

69. Doyon, Y., Selleck, W., Lane, W.S., Tan, S., and Cote, J. (2004). Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans. Mol Cell Biol *24*, 1884-1896.

70. Ikura, T., Ogryzko, V.V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J., and Nakatani, Y. (2000). Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell *102*, 463-473.

71. Ramsay, R.G., and Gonda, T.J. (2008). MYB function in normal and cancer cells. Nat Rev Cancer *8*, 523-534.

72. Miglarese, M.R., Richardson, A.F., Aziz, N., and Bender, T.P. (1996). Differential regulation of c-Myb-induced transcription activation by a phosphorylation site in the negative regulatory domain. J Biol Chem *271*, 22697-22705.

73. Karin, M. (2009). NF-kappaB as a critical link between inflammation and cancer. Cold Spring Harb Perspect Biol *1*, a000141.

74. Pazolli, E., Luo, X., Brehm, S., Carbery, K., Chung, J.J., Prior, J.L., Doherty, J., Demehri, S., Salavaggione, L., Piwnica-Worms, D., and Stewart, S.A. (2009). Senescent stromal-derived osteopontin promotes preneoplastic cell growth. Cancer Res *69*, 1230-1239.

75. Coppe, J.P., Patil, C.K., Rodier, F., Krtolica, A., Beausejour, C.M., Parrinello, S., Hodgson, J.G., Chin, K., Desprez, P.Y., and Campisi, J. A human-like senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen. PLoS One *5*, e9188.

Appendix 1: Myb as a Putative Transcriptional Regulator of OPN in Senescence

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E.Pazolli and E.Oster were equal contributors.

Introduction and Results

Early studies on the c-Myb transcription factor led to its designation as a hematopoietic lineage determinant [1]. However, further work demonstrated that Myb participates in cell cycle progression, differentiation and oncogenesis ([2] and references therein). Myb affects the expression of its target genes via direct binding to its cognate sequence and/or interactions with specific partners including C/EBPβ [3]. Recently, it was shown that c-Myb protein levels increase in late passage human fibroblasts possibly contributing to enhanced protection against oxidative stress [4]. In Chapter 3, I identified a fragment of the human OPN promoter that was consistently stimulated in senescence (**Figure 3.6A**). This fragment contains the sequence TAACTGT, which is a canonical Myb-binding site (MBS) [5]. Reportedly, c-Myb binds directly to the human OPN promoter and drives its expression in metastatic melanoma cell lines [6]. In this Appendix, I describe preliminary experiments that suggest a role for Myb in the transcriptional activation of OPN in senescence.

To test the functionality of the MBS in the OPN promoter, we performed co-transfection experiments. Young and senescent fibroblasts transiently transfected with OPN promoter fragment containing the MBS (OPN190) and c-Myb demonstrated a robust induction of promoter activity arguing that c-Myb is a limiting factor in OPN transcription (**Figure A1.1**). Next, we substituted several core nucleotides as well as surrounding ones (labeled as MBS-mut), to determine the specificity of the sequence as a target of endogeneous c-Myb binding. In line with the previous experiment, alteration of

the core binding sequence abrogated activity of the reporter in senescent fibroblasts strongly suggesting that this sequence is required for Myb binding *in vivo* (**Figure A1.2**). To further examine the role of Myb, I employed lentiviral-based short hairpins to reduce its levels in senescent fibroblasts. Upon RNAi-mediated depletion of c-Myb, we observed a significant decline in OPN levels (**Figure A1.3**). To further validate these results, we have obtained additional short hairpins that target Myb and are currently investigating whether Myb binds the endogeneous OPN promoter in senescence utilizing chromatin immunoprecipitations (ChIP). In conclusion, these results suggest that Myb binds the OPN promoter in a sequence-specific manner and is required for OPN upregulation in senescence.

A1.2 Discussion and Conclusions

To our knowledge, this is the first instance implicating Myb in the regulation of OPN transcription in senescent fibroblasts. Although, it was reported that Myb protein levels increase in late passage fibroblasts, we did not observe any differences between young and senescent fibroblasts (**Figure A1.4**) [4]. This could be attributed to the mode of senescence induction (stress-induced premature senescence versus replicative senescence) or the cell lines used. At present, it is unclear how Myb's activity in senescence is regulated. Notably, Myb is subject to a variety of posttranslational modifications [2], which can affect its DNA binding ability and/or transactivation activity without altering protein levels. p42(MAPK)-mediated phosphorylation of c-Myb differentially modifies its transactivation activity in a promoter-specific manner [7].

Likewise, acetylation of the Myb negative regulatory domain by CREB-binding protein (CBP) enhances its affinity for CBP and Myb's transactivation capacity [8]. There is evidence for altered mitogenic signaling [9, 10] and global chromatin remodeling [11] in senescence, which may lay the ground for differential activation of transcription factors and we have shown (see Chapter 3) that chromatin is a central driver of SASP expression. Examination of possible Myb modifications in our system may shed light on the regulatory mechanisms invoked in senescence.

As mentioned above, c-Myb cooperates with multiple transcription factors and regulatory complexes, which facilitates customized transcriptional responses. A classical example is the interaction with CCAAT/Enhancer Binding Protein β (C/EBPβ) on the established Myb-inducible *mim-1* promoter [5]. Ectopic expression of both transcription factors stimulates the full activity of the *mim-1* promoter in fibroblasts, where it normally is suppressed [12]. Furthermore, the synergistic effect is attributed to chromatin relaxation and a long range interaction over an enhancer element located approximately 2kb upstream of the Myb-regulated boxes in the promoter [13, 14]. The OPN promoter contains a cluster of C/EBPβ sites positioned at a similar distance from the MBS characterized herein. It is noteworthy that C/EBPβ is overexpressed in senescence and has been implicated in the transcriptional activation of SASP [15], which raises the possibility of combinatorial switches driving senescence-associated OPN expression. However, preliminary results suggest that C/EBPβ is not involved in OPN upregulation in senescence, in contrast to IL6 and IL8. Ectopic expression of the LIP isoform, which blocks C/EBPβ-driven transcriptional responses in a dominant negative fashion [16, 17],

has no effect on OPN levels in senescence, while it abrogates the concomitant IL6 and IL8 upregulation (data not shown). Myb can partner with other transcription factors however [2]; therefore, it will be interesting to uncover interactions important for OPN transcription in senescence.

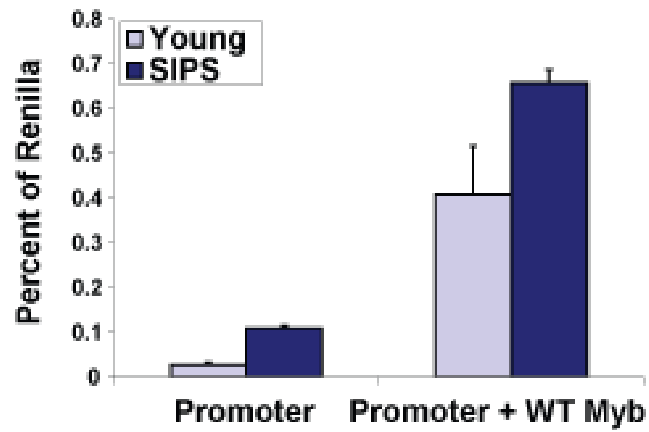

Figure A1.1 c-Myb activates the OPN promoter. Young and senescent fibroblasts were transiently transfected with the OPN190 fragment driving luciferase (left) and WT c-Myb (right). Luciferase levels were measured after 48 hours and expressed as percent of pGL3-Renilla, used as a control for transfection efficiency (n=3).

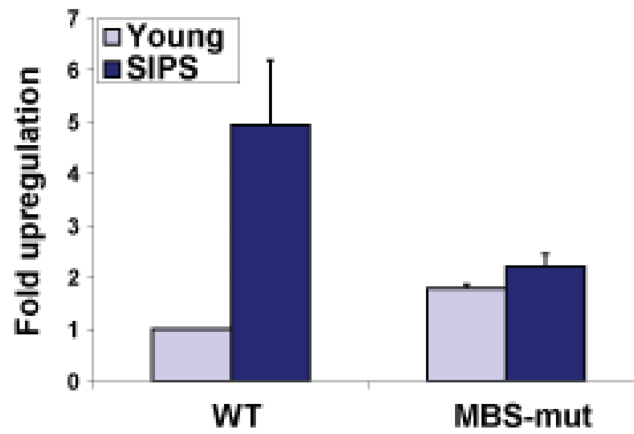

Figure A1.2 The MBS in the OPN promoter is functional. Young and senescent fibroblasts were transiently transfected as in Figure A1.1 with the OPN190 fragment containing the canonical MBS or OPN190 with the underlined residues mutated from TAACTGTAGAT to TGCTAGTAGAC. Readings were performed as described earlier (n=2).

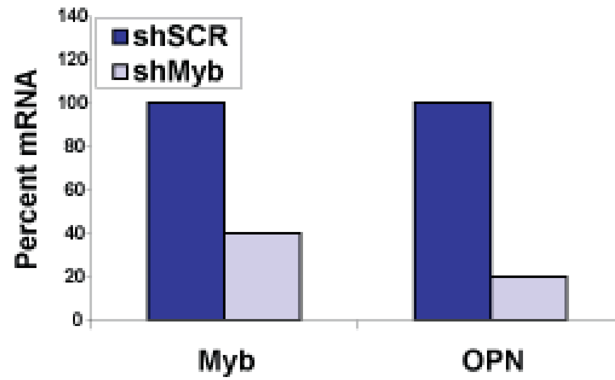

Figure A1.3 RNAi-directed depletion of c-Myb decreases OPN levels in senescence. Senescent BJ fibroblasts expressing a hairpin targeting Myb (causes a 60% reduction in Myb levels) have lower OPN expression when compared to senescent cells expressing a control hairpin (shSCR set at 100%) (n=3).

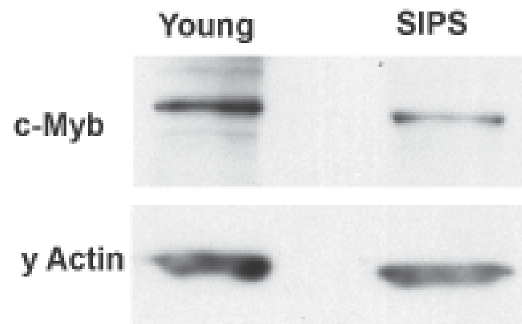

Figure A1.4 No significant differences in Myb levels in young and senescent fibroblasts. Lysates of young and senescent BJ fibroblasts were probed with an antibody against c-Myb and γ Actin as a loading control.

References

1. Mucenski, M.L., McLain, K., Kier, A.B., Swerdlow, S.H., Schreiner, C.M., Miller, T.A., Pietryga, D.W., Scott, W.J., Jr., and Potter, S.S. (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell *65*, 677-689.
2. Ramsay, R.G., and Gonda, T.J. (2008). MYB function in normal and cancer cells. Nat Rev Cancer *8*, 523-534.
3. Burk, O., Mink, S., Ringwald, M., and Klempnauer, K.H. (1993). Synergistic activation of the chicken mim-1 gene by v-myb and C/EBP transcription factors. Embo J *12*, 2027-2038.
4. Lee, Y.H., Lee, N.H., Bhattarai, G., Hwang, P.H., Kim, T.I., Jhee, E.C., and Yi, H.K. c-myb has a character of oxidative stress resistance in aged human diploid fibroblasts: regulates SAPK/JNK and Hsp60 pathway consequently. Biogerontology *11*, 267-274.
5. Ness, S.A., Marknell, A., and Graf, T. (1989). The v-myb oncogene product binds to and activates the promyelocyte-specific mim-1 gene. Cell *59*, 1115-1125.
6. Schultz, J., Lorenz, P., Ibrahim, S.M., Kundt, G., Gross, G., and Kunz, M. (2009). The functional -443T/C osteopontin promoter polymorphism influences osteopontin gene expression in melanoma cells via binding of c-Myb transcription factor. Mol Carcinog *48*, 14-23.
7. Miglarese, M.R., Richardson, A.F., Aziz, N., and Bender, T.P. (1996). Differential regulation of c-Myb-induced transcription activation by a phosphorylation site in the negative regulatory domain. J Biol Chem *271*, 22697-22705.
8. Sano, Y., and Ishii, S. (2001). Increased affinity of c-Myb for CREB-binding protein (CBP) after CBP-induced acetylation. J Biol Chem *276*, 3674-3682.
9. Courtois-Cox, S., Genther Williams, S.M., Reczek, E.E., Johnson, B.W., McGillicuddy, L.T., Johannessen, C.M., Hollstein, P.E., MacCollin, M., and Cichowski, K. (2006). A negative feedback signaling network underlies oncogene-induced senescence. Cancer Cell *10*, 459-472.
10. Nogueira, V., Park, Y., Chen, C.C., Xu, P.Z., Chen, M.L., Tonic, I., Unterman, T., and Hay, N. (2008). Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis. Cancer Cell *14*, 458-470.
11. Oberdoerffer, P., and Sinclair, D.A. (2007). The role of nuclear architecture in genomic instability and ageing. Nat Rev Mol Cell Biol *8*, 692-702.

12. Ness, S.A., Kowenz-Leutz, E., Casini, T., Graf, T., and Leutz, A. (1993). Myb and NF-M: combinatorial activators of myeloid genes in heterologous cell types. Genes Dev *7*, 749-759.

13. Plachetka, A., Chayka, O., Wilczek, C., Melnik, S., Bonifer, C., and Klempnauer, K.H. (2008). C/EBPbeta induces chromatin opening at a cell-type-specific enhancer. Mol Cell Biol *28*, 2102-2112.

14. Tahirov, T.H., Sato, K., Ichikawa-Iwata, E., Sasaki, M., Inoue-Bungo, T., Shiina, M., Kimura, K., Takata, S., Fujikawa, A., Morii, H., Kumasaka, T., Yamamoto, M., Ishii, S., and Ogata, K. (2002). Mechanism of c-Myb-C/EBP beta cooperation from separated sites on a promoter. Cell *108*, 57-70.

15. Kuilman, T., Michaloglou, C., Vredeveld, L.C., Douma, S., van Doorn, R., Desmet, C.J., Aarden, L.A., Mooi, W.J., and Peeper, D.S. (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell *133*, 1019-1031.

16. Descombes, P., and Schibler, U. (1991). A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell *67*, 569-579.

17. Gomis, R.R., Alarcon, C., Nadal, C., Van Poznak, C., and Massague, J. (2006). C/EBPbeta at the core of the TGFbeta cytostatic response and its evasion in metastatic breast cancer cells. Cancer Cell *10*, 203-214.

Appendix 2: TGFβ-independent OPN Regulation in Senescence

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E. Pazolli was the principal contributor to this work.

Introduction

Transforming growth factor β (TGF-β) signaling is multifaceted and results in diverse physiological outcomes depending on the cellular context. Generally, ligands of the super TGF-β family bind and activate a complex consisting of type I and II receptors, which phosphorylates the receptor-regulated Smads (R-Smads) at the SSXS motif in the C-terminus. Upon activation, R-Smads disassociate from the receptor, heteromerize with the Co-mediator Smad (Smad4) and translocate into the nucleus where they drive transcription in cooperation with other nuclear factors [1]. The linear pathway delineated above comprises classical TGF-β-dependent signaling; however, R-Smads respond to stimuli originating from other pathways in the cell including the mitogen activated protein kinases MEK, p38 and JNK, which in a context-dependent manner can either synergize, antagonize or operate independently of TGF-β [2]. TGF-β cytostatic responses are well characterized; hence its designation as a tumor-suppressor in multiple systems [3]. Consequently, the pathway, and specifically Smad3 – an R-Smad, has been implicated in the regulation of senescence [4-6]. Furthermore, the TGFβ pathway coordinates the expression of several genes during wound repair [7], a similar profile to that encountered in senescence [8]. Interestingly, c-Myb is involved in the execution of wound healing and its deficiency leads to impaired Smad3 activation during this process [9] raising the possibility that these two pathways cooperate.

The senescent-associated transcriptome is enriched in proteins classified as components of an extracellular matrix and growth factor cluster [10] reminiscent of a

wounding profile [11]. Noticeably, there is a subset of bone fide TGFβ targets in our microarray analysis that are upregulated in senescent fibroblasts, which is a key remodeling orchestrator during wound healing (data not shown). Taking a closer look at the OPN promoter, I observed a cluster of Smad-binding elements (SBE) [12] distributed throughout and in close proximity to the region of interest described above (**Figure A2.1A**). Therefore it is plausible that Smad3, a central transducer of TGFβ signals is involved in the regulation of OPN in senescence.

Results and Conclusions

To test the above hypothesis, I examined OPN levels in fibroblasts with a successful knockdown of Smad3 (**Figure A2.1B**). In my original analysis, three short hairpins resulted in 87, 83, and 96 percent knockdown of Smad3. OPN levels in these cells were reduced by 62, 90, and 89 percent respectively, suggesting that Smad3 regulated OPN expression in senescent fibroblasts. Unfortunately, in subsequent experiments we were only able to deplete Smad3 with one of the three original RNAi constructs, which maintained the effect on OPN expression (**Figure A2.1C**). Therefore, to interrogate the putative involvement of Smad3 activity on OPN expression I obtained an additional short hairpin, encoded by a different vector (pRS-shSmad3). Using pRS-shSmad3, I achieved a slightly superior knockdown in Smad3 protein compared to the original short hairpin (**Figure A2.1B**). Analysis of OPN mRNA levels in the pRS-shSmad3 expressing cells did not reveal any significant loss of OPN expression (**Figure**

A2.1C) raising the possibility that our earlier results were an off-target effect of the Smad3 hairpin.

Given the inconclusive nature of the RNAi experiments, I interrogated the putative implication of the TGFβ pathway in OPN expression in senescence via pharmacologic inhibitors and genetic tools. The well characterized classical TGFβ pathway relies on receptor-mediated activation of Smads – upon ligand binding, TGFβ type II receptor (TβRII) phosphorylates the type I receptor (TβRI), which in turn activates Smads [1]. Disruption of TβRI activity by a specific and potent inhibitor, SB-431542 [13] successfully blocked TGFβ-induced plasminogen activator inhibitor type 1 (PAI-1) activation, which is a classic TGFβ-responsive gene [14] (**Figure A2.2A**); however, upon induction of senescence, OPN levels increased in a typical fashion and there was no impact on OPN expression in the presence of the inhibitor (**Figure A2.2B**). Similarly, ectopic expression of a cytoplasmically truncated TβRII ([15] and Addgene plasmid 12640), which acts in a dominant-negative fashion (**Figure A2.2C**), did not have an impact on OPN upregulation (**Figure A2.2D**). To corroborate the absence of receptor involvement, I employed a Smad3 construct that lacks the last four amino acids in the C-terminus (Smad3deltaC), which are indispensable for receptor-mediated phosphorylation ([15] and Addgene plasmid 12639). Upon senescence induction in the Smad3deltaC-overexpressing cells, the robust activation of OPN remained unperturbed (**Figure A2.3A**). Together these results indicate that OPN expression in senescence is not subject to canonical TGFβ-induced signaling. This is further substantiated by the lack of

detectable differences in C-terminus phosphorylated Smad3 and TFGβ levels between young and senescent fibroblasts (**Figure A2.3B** and **C**).

R-Smads have a proline-rich linker region that connects the well-conserved MH1 and MH2 domains [1]. Initially, multiple phosphorylation sites were suspected in the linker based on its sequence. Several reports have now confirmed that the MAPK pathways (ERK, p38 and JNK) [16-18] and other kinases [19-21] directly phosphorylate Smad3 and affect its function. In most settings, noncanonical phosphorylation of Smad3 channels the input of multiple signaling pathways into TGFβ-driven responses; however, this does not preclude TGFβ-independent activation of such nonreceptor kinases and subsequent Smad activation [2]. Given that Smad3 appears to operate independently from TGFβ receptor-mediated signaling in our system, I examined whether other pathways implicated in its activation, were involved. When senescent fibroblasts were incubated with chemical inhibitors targeting MEK (U0126) [22], p38 (SB 203580) [23] and JNK (SP600125) [24] either separately on in combination, we observed a significant decrease in OPN levels (**Figure A2.4A**) suggesting that MAPK signaling influences OPN upregulation in senescence. In agreement with this, we observed differential phosphorylation of the linker region of Smad3 in senescent fibroblasts. There is a modest increase in phosphorylation of residues S204 and S208 in senescent fibroblasts, which are known targets of MAPKs (data not shown).

To test whether these specific phosphorylated residues are important for OPN expression in senescence, we obtained a Smad3 construct that is mutated in all four residues implicated in MAPK signaling labeled as Smad3-EPSM [25]. Ectopic

expression of the Smad3-EPSM construct had no effect on OPN expression in senescence arguing that these residues are not required for signal transduction in senescence. Finally, to rule out any involvement of the Smad pathway, we obtained short hairpins targeting Smad4, usually required for Smad3-mediated signaling [1]. Despite a significant depletion of Smad4 with three independent hairpins knocking down between 60-80%, OPN levels were comparable to the control cells (**Figure A2.5**). In conclusion, our results indicate that the TGFβ pathway is not involved in the regulation of OPN transcription in senescence.

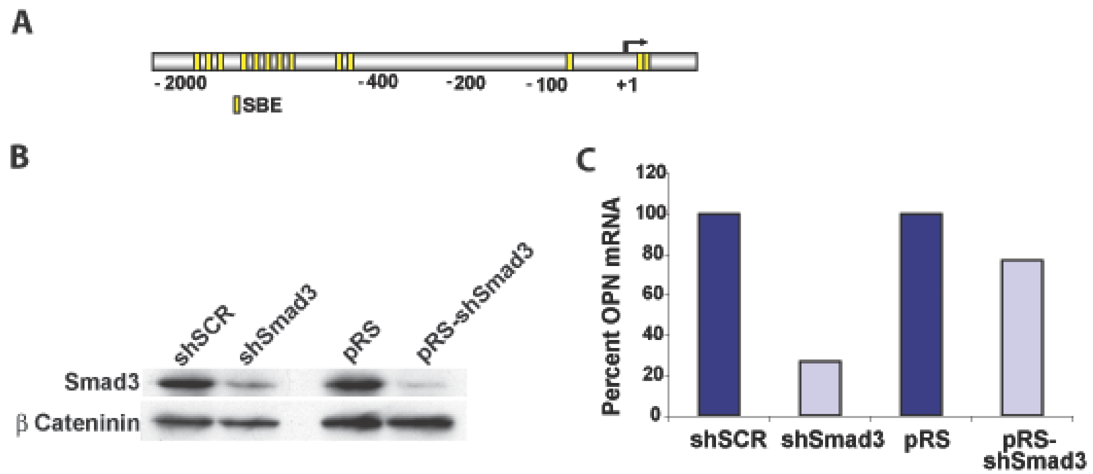

Figure A2.1 RNAi-mediated depletion of Smad3 in senescent fibroblasts. A, The human OPN promoter contains multiple Smad-binding elements (SBE) clustered together [26]. B, Smad3 protein levels were compared by Western Blot analysis in BJ fibroblasts expressing a control hairpin (shSCR) or control vector (pRS) to cells expressing hairpins targeting Smad3. β-Catenin was used as a loading control. C, OPN mRNA levels were determined by quantitative RT-PCR (qRT-PCR) in the same cells as in B (n=2).

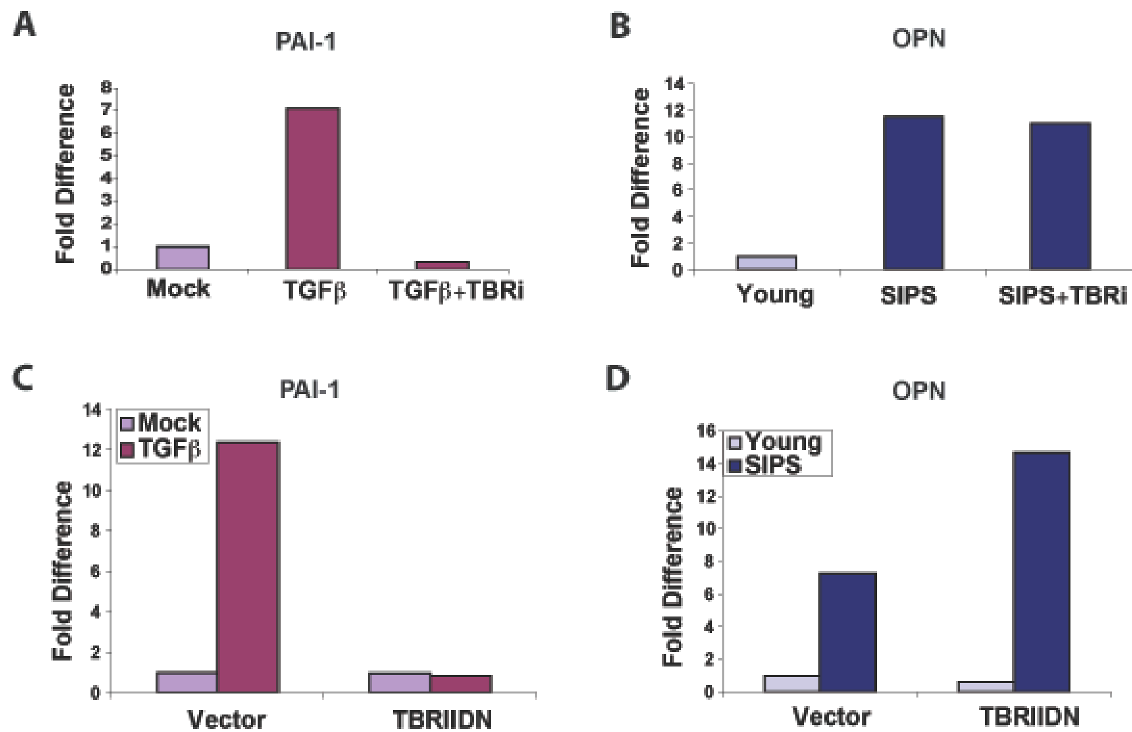

Figure A2.2 TGFβ receptor-mediated signaling is dispensable for OPN expression. A, BJ fibroblasts were treated with 10ng/ml of TGFβ in the presence of vehicle or a specific inhibitor (TBRi) for 48 hrs and PAI-1 mRNA levels were quantitated by qRT-PCR. B, Senescent fibroblasts were treated with TBRi and OPN mRNA levels were quantitated by qRT-PCR (n=4). C, BJ fibroblasts transduced with a dominant negative allele of TGFβ receptor type II (TBRIIDN) were treated with TGFβ as in A and PAI-1 levels were measured by qRT-PCR. D, Senescence was induced in the same cells as in C and OPN mRNA levels were measured by qRT-PCR (n=2).

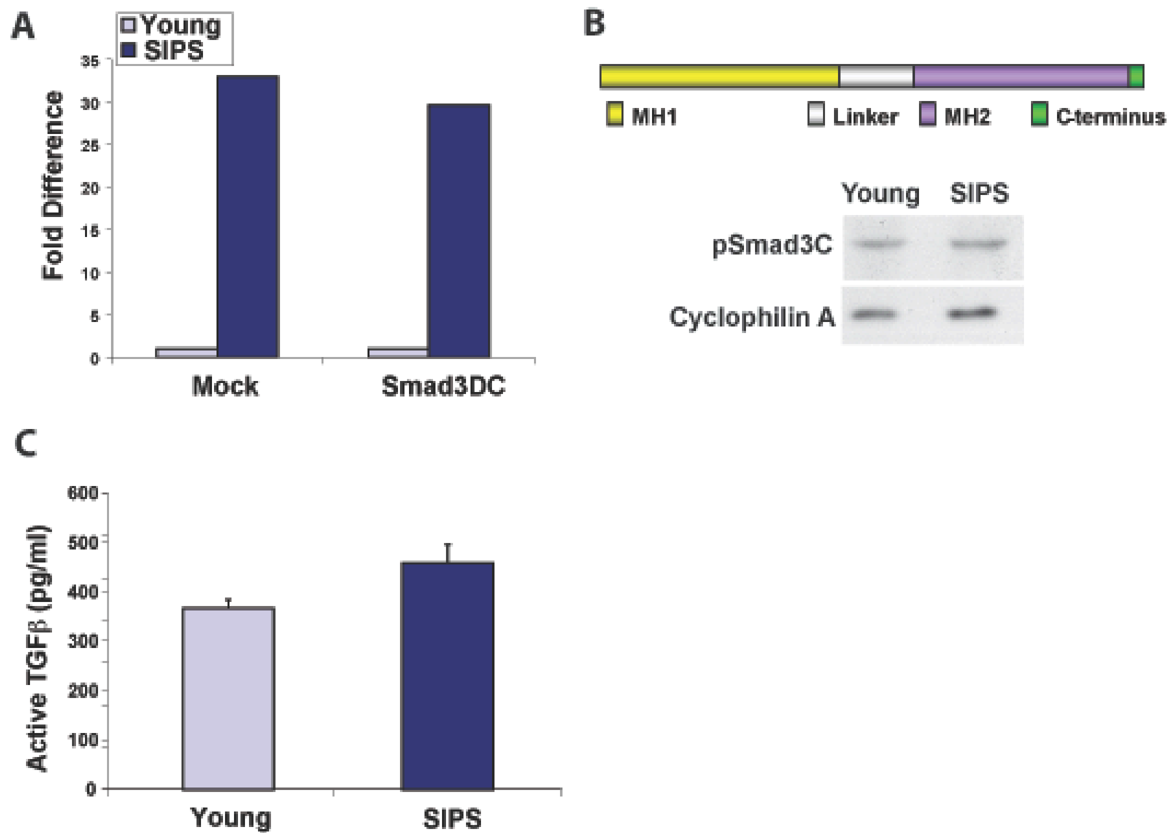

Figure A2.3 TGFβ canonical signaling does not affect OPN expression in senescence. A, BJ fibroblasts were transduced with a dominant negative allele of Smad3 that lacks four amino acids in the C terminus. OPN levels were measured by qRT-PCR in young and senescent cells (n=2). B, Smad3 consists of two major domains, MH1 and MH2 separated by a linker region. pSmad3C recognizes the TGFβ-mediated phosphorylation in the C terminus residues. Cyclophilin A was used as a loading control. C, TGFβ-1 levels were measured by an ELISA in young and senescent BJ fibroblasts (n=2).

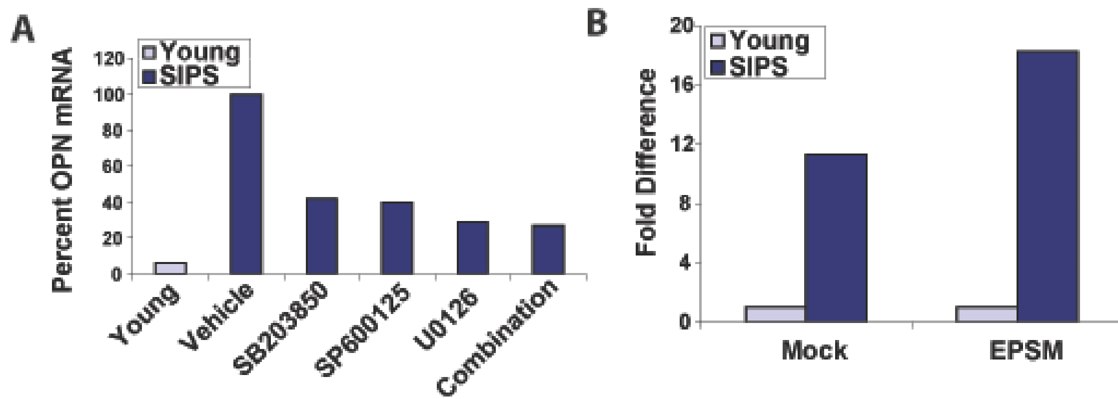

Figure A2.4 The linker region is not necessary for OPN activation in senescence. A, Senescent cells were incubated with different inhibitors individually or in combination (see text) for 72 hr. OPN mRNA levels were measured by qRT-PCR (n=3). B, OPN mRNA levels were measured in young and senescent BJ fibroblasts transduced with a point mutant Smad3 (see text) (n=2).

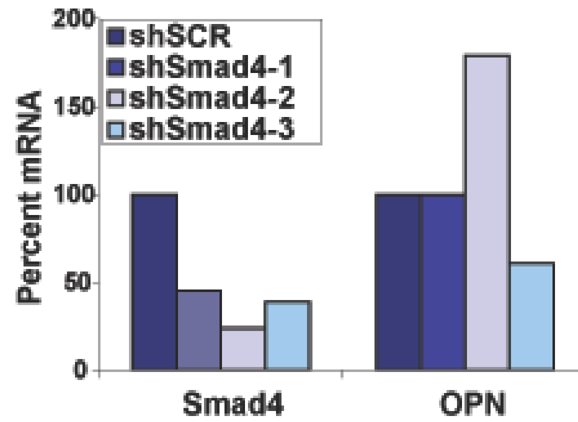

Figure A2.5 Smad4 depletion has no effect on OPN expression. Senescent fibroblasts expressing three independent short hairpins targeting Smad4 (left) were examined for OPN levels by qRT-PCR (right) (n=2).

References

1. Shi, Y., and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell *113*, 685-700.
2. Javelaud, D., and Mauviel, A. (2005). Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis. Oncogene *24*, 5742-5750.
3. Biswas, S., Criswell, T.L., Wang, S.E., and Arteaga, C.L. (2006). Inhibition of transforming growth factor-beta signaling in human cancer: targeting a tumor suppressor network as a therapeutic strategy. Clin Cancer Res *12*, 4142-4146.
4. Passos, J.F., Nelson, G., Wang, C., Richter, T., Simillion, C., Proctor, C.J., Miwa, S., Olijslagers, S., Hallinan, J., Wipat, A., Saretzki, G., Rudolph, K.L., Kirkwood, T.B., and von Zglinicki, T. Feedback between p21 and reactive oxygen production is necessary for cell senescence. Mol Syst Biol *6*, 347.
5. Pascal, T., Debacq-Chainiaux, F., Chretien, A., Bastin, C., Dabee, A.F., Bertholet, V., Remacle, J., and Toussaint, O. (2005). Comparison of replicative senescence and stress-induced premature senescence combining differential display and low-density DNA arrays. FEBS Lett *579*, 3651-3659.
6. Vijayachandra, K., Lee, J., and Glick, A.B. (2003). Smad3 regulates senescence and malignant conversion in a mouse multistage skin carcinogenesis model. Cancer Res *63*, 3447-3452.
7. Klass, B.R., Grobbelaar, A.O., and Rolfe, K.J. (2009). Transforming growth factor beta1 signalling, wound healing and repair: a multifunctional cytokine with clinical implications for wound repair, a delicate balance. Postgrad Med J *85*, 9-14.
8. Shelton, D.N., Chang, E., Whittier, P.S., Choi, D., and Funk, W.D. (1999). Microarray analysis of replicative senescence. Curr Biol *9*, 939-945.
9. Kopecki, Z., Luchetti, M.M., Adams, D.H., Strudwick, X., Mantamadiotis, T., Stoppacciaro, A., Gabrielli, A., Ramsay, R.G., and Cowin, A.J. (2007). Collagen loss and impaired wound healing is associated with c-Myb deficiency. J Pathol *211*, 351-361.
10. Pazolli, E., Luo, X., Brehm, S., Carbery, K., Chung, J.J., Prior, J.L., Doherty, J., Demehri, S., Salavaggione, L., Piwnica-Worms, D., and Stewart, S.A. (2009). Senescent stromal-derived osteopontin promotes preneoplastic cell growth. Cancer Res *69*, 1230-1239.
11. Chang, H.Y., Sneddon, J.B., Alizadeh, A.A., Sood, R., West, R.B., Montgomery, K., Chi, J.T., van de Rijn, M., Botstein, D., and Brown, P.O. (2004). Gene

expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. PLoS Biol *2*, E7.

12. Jonk, L.J., Itoh, S., Heldin, C.H., ten Dijke, P., and Kruijer, W. (1998). Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-beta, activin, and bone morphogenetic protein-inducible enhancer. J Biol Chem *273*, 21145-21152.

13. Inman, G.J., Nicolas, F.J., Callahan, J.F., Harling, J.D., Gaster, L.M., Reith, A.D., Laping, N.J., and Hill, C.S. (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol *62*, 65-74.

14. Lund, L.R., Riccio, A., Andreasen, P.A., Nielsen, L.S., Kristensen, P., Laiho, M., Saksela, O., Blasi, F., and Dano, K. (1987). Transforming growth factor-beta is a strong and fast acting positive regulator of the level of type-1 plasminogen activator inhibitor mRNA in WI-38 human lung fibroblasts. Embo J *6*, 1281-1286.

15. Choy, L., Skillington, J., and Derynck, R. (2000). Roles of autocrine TGF-beta receptor and Smad signaling in adipocyte differentiation. J Cell Biol *149*, 667-682.

16. Engel, M.E., McDonnell, M.A., Law, B.K., and Moses, H.L. (1999). Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. J Biol Chem *274*, 37413-37420.

17. Matsuura, I., Wang, G., He, D., and Liu, F. (2005). Identification and characterization of ERK MAP kinase phosphorylation sites in Smad3. Biochemistry *44*, 12546-12553.

18. Hayes, S.A., Huang, X., Kambhampati, S., Platanias, L.C., and Bergan, R.C. (2003). p38 MAP kinase modulates Smad-dependent changes in human prostate cell adhesion. Oncogene *22*, 4841-4850.

19. Wicks, S.J., Lui, S., Abdel-Wahab, N., Mason, R.M., and Chantry, A. (2000). Inactivation of smad-transforming growth factor beta signaling by Ca(2+)-calmodulin-dependent protein kinase II. Mol Cell Biol *20*, 8103-8111.

20. Long, J., Wang, G., Matsuura, I., He, D., and Liu, F. (2004). Activation of Smad transcriptional activity by protein inhibitor of activated STAT3 (PIAS3). Proc Natl Acad Sci U S A *101*, 99-104.

21. Alarcon, C., Zaromytidou, A.I., Xi, Q., Gao, S., Yu, J., Fujisawa, S., Barlas, A., Miller, A.N., Manova-Todorova, K., Macias, M.J., Sapkota, G., Pan, D., and Massague, J. (2009). Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. Cell *139*, 757-769.

22. Duncia, J.V., Santella, J.B., 3rd, Higley, C.A., Pitts, W.J., Wityak, J., Frietze, W.E., Rankin, F.W., Sun, J.H., Earl, R.A., Tabaka, A.C., Teleha, C.A., Blom, K.F., Favata, M.F., Manos, E.J., Daulerio, A.J., Stradley, D.A., Horiuchi, K., Copeland, R.A., Scherle, P.A., Trzaskos, J.M., Magolda, R.L., Trainor, G.L., Wexler, R.R., Hobbs, F.W., and Olson, R.E. (1998). MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. Bioorg Med Chem Lett *8*, 2839-2844.

23. Cuenda, A., and Cohen, P. (1999). Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. J Biol Chem *274*, 4341-4346.

24. Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., and Anderson, D.W. (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A *98*, 13681-13686.

25. Kretzschmar, M., Doody, J., Timokhina, I., and Massague, J. (1999). A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. Genes Dev *13*, 804-816.

26. Zawel, L., Dai, J.L., Buckhaults, P., Zhou, S., Kinzler, K.W., Vogelstein, B., and Kern, S.E. (1998). Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cell *1*, 611-617.