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Extra View

# Senescence, Wound Healing and Cancer

## The PAI-1 Connection

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**KEY WORDS**

senescence, stroma, metastasis, wound healing, p53, Plasminogen activator inhibitor 1, uPA, fibroblast, growth factor

**ABBREVIATIONS**

| | |
|---|---|
| PAI-1 | plasminogen activator inhibitor-1 |
| uPA | urokinase-type plasminogen activator |
| pRb | retinoblastoma protein |
| MEF | mouse embryo fibroblast |
| CDK | cyclin dependent kinase |
| ECM | extracellular matrix |
| TGFβ | transforming growth factor β |
| HIF1α | hypoxia inducible factor 1α |
| MMP | matrix metalloproteinase |
| EGF | epidermal growth factor |
| bFGF | basic-fibroblast growth factor or FGF2 |
| HGF | hepatocyte growth factor |
| SF | scatter factor |
| IGF | insulin-like growth factor |
| UPAR | urokinase-type plasminogen activator receptor |
| LDL | low density lipoprotein |
| Kd | knockdown |
| PI3K | phosphoinositide 3-kinase |
| PKB | protein kinase B or AKT |

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## ABSTRACT

Prolonged propagation of primary diploid fibroblasts in culture activates an ageing process known as replicative senescence, which is considered to provide a barrier against oncogenic transformation. Remarkably, both cell autonomous tumor-suppressive and cell nonautonomous tumor-promoting effects of senescent cells have been reported. Recently, we described that the p53 target gene plasminogen activator inhibitor-1 (*PAI-1*) is an essential mediator of replicative senescence. PAI-1 antagonizes the protease urokinase-type plasminogen activator (uPA). Both are secreted factors and involved in heterotypic signaling processes such as wound healing, angiogenesis and metastasis. Both uPA and PAI-1 are expressed in senescent cells and their relative abundance controls proliferation downstream of p53. Here, we present data that the effects of PAI-1 and uPA in the senescence response are not strictly cell autonomous. We discuss these findings in the context of the emerging roles of PAI-1 and uPA in heterotypic cellular signaling in senescence, wound healing and metastasis.

## INTRODUCTION

Cellular senescence can be regarded as the physiological end-state of the proliferative capacity of cells. When cells are cultured, they progressively cease to proliferate over time, become unresponsive to mitogenic stimuli, change morphologically, and, though they are in a stable $G_1$ cell cycle arrest, remain viable. This phenomenon was first recognized by Hayflick in 1961[1] and is thought to protect cells from uncontrolled proliferation. Senescence is the result of a stress-response program in which various stress signals accumulate to activate the tumor-suppressive effects of the pRb and p53 pathways.[2] Therefore, new insights into the function of many fundamentally critical cancer-related genes have been obtained by studying replicative senescence in fibroblasts.[3,4] Recently, it was confirmed that both in mouse and human tumorigenesis senescence is a bona fide tumor suppressive mechanism in vivo.[5-8] However, multiple key issues of the senescence response remain unresolved. First, how does p53 contribute to the induction of senescence? There is a plethora of data describing how various stress-related pathways activate p53.[2,9-11] But what happens downstream of p53? Second, how can senescent cells, which themselves do not proliferate, stimulate proliferation of adjacent cells?[12,13] Why do senescent cells have these two faces? We provide evidence that plasminogen activator inhibitor-1 might be a central player in both questions, and reflect on possible implications of these observations for our understanding of fibroblast behavior, wound healing, and metastasis.

## THE MOLECULAR BASIS OF SENESCENCE

Primary murine cells activate the $p19^{ARF}$-p53 tumor suppressor pathway during prolonged culturing in vitro, inducing an arrest in the $G_1$ phase of the cell cycle.[4,14] This senescence response reflects a fail-safe mechanism that acts to protect cells from aberrant growth and oncogenic transformation in vivo.[9,15] Senescence can be overcome by loss of *$p19^{ARF}$*, *p53*, or all three retinoblastoma (Rb) family proteins, collectively known as the "pocket proteins" (pRb, p107, and p130), which results in proliferation due to deregulated E2F activity.[3,4,10] In human cells the tumor-suppressor $p16^{INK4A}$ and telomere-erosion also play roles in senescence-induction.[9,16] The tumor-suppressive functions of p53 and pRb can also be abrogated by various viral and cellular oncogenes, which render cells insensitive to a variety of mitogenic, anti-proliferative, DNA damage, and oxidative stress signals.[2] In normal fibroblasts, proliferation is induced by extra-cellular growth factors. They stimulate mitogenic signaling leading to activation of cyclin-dependent kinases (CDKs), which

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in turn inactivate pRb's growth-inhibitory activity. Such mitogenic signaling is essential for cells to pass the pRb-controlled $G_1$ cell cycle checkpoint, which is often deregulated in cancer.[4,10] E2F transcription factors are essential downstream components in the p53-induced $G_1$ arrest.[17] A tumor-suppressive function of p53 might therefore be to block growth factor signaling to the pocket proteins. However, mouse embryo fibroblasts (MEFs) knockout for *p21$^{CIP1}$*, a p53 target gene and potent inhibitor of CDK activity, are not immortal.[18] A link between p53 and mitogenic signaling to pRb is nevertheless suspected, since MEFs lacking all three pocket proteins are immortal in the presence of a functional p53 pathway[19] and a dominant negative mutant of E2F (E2F-DB) immortalizes MEFs in the presence of an activated p19$^{ARF}$-p53 pathway.[17] How p53 communicates with pRb to induce a proliferation arrest is not well understood and it is therefore unclear how cells become insensitive to p53 activity by deregulated mitogenic signaling. Furthermore, it is also not understood whether enhanced growth factor signaling can be a direct consequence of p53 loss. To shed light on the suspected link between growth factor signaling and p53, we discuss below three physiological responses of fibroblasts: the serum response, wound healing and senescence.

## FIBROBLASTS IN WOUND HEALING AND SENESCENCE

Recent studies have elegantly shown that the reaction of primary fibroblasts to serum induces an inflammatory response, leaving the fibroblasts in an activated state.[20-22] Micro-array analysis of fibroblast proliferation induced by serum-addition shows a gene-signature resembling that of an activated fresh wound,[20] and this wound-like profile is a poor prognosis marker for multiple types of adenocarcinomas.[22,23] Interestingly, it was noted already some time ago that both inflammation and wound-healing might be linked to cancer.[24-26] Over the past years these links have been substantiated by showing that molecules involved in inflammation and wound healing are causally involved in cancer.[25,27-31] Fibroblasts play a causal role in wound healing, which in the initial phase is driven by heterotypic signaling between fibroblasts and immune system cells recruited during inflammation, such as platelets, macrophages, leukocytes and mast cells.[32,33] During cutaneous wound healing, following activation by inflammatory molecules, fibroblasts proliferate and migrate into the open wound, deposit a provisional fibrin layer, and stop dividing or become necrotic.[32,34,35] There are several similarities in the molecular events that take place in fibroblasts in response to serum and during ageing, wound healing, and the induction of replicative senescence. For example: (1) mouse models show that healing wounds depend on fibroblasts for closure,[33,36] (2) chronic human wounds contain fibroblasts with diminished or absent replicative potential, and this seems to be telomere-erosion independent[37-39] (3) senescence in vitro is regarded premature ageing of cells,[1,40] (4) fibroblasts of elderly people have shorter replicative potential when cultured,[41] (5) increased age is correlated with poor prospects for healing of a wound,[42] (6) there is presence of senescent cells in age-related pathologies,[43] and (7) the number of senescent fibroblasts increases exponentially in the skin of ageing primates.[44] So, which genes might be involved in all these processes and can candidate genes involved in serum-induced activation of a fibroblast and wound healing possibly also be involved in senescence?

## PAI-1 REGULATION IN FIBROBLASTS

To investigate the relationship between serum stimulation, wound healing and senescence, we have in the recent past focused our attention to PAI-1. Our interest in this gene was sparked by several observations:

(1) p53 and PAI-1 regulation during the wound healing response in vivo is similar to what is observed during activation of a fibroblast and senescence in vitro: There is sequential upregulation of the activity of both over time, with senescent cells and closing wounds having the highest levels of p53 and PAI-1.[45-48]

(2) PAI-1 is a senescence marker in fibroblasts, both in vivo and in vitro.[46,49]

(3) p53 is critically involved in the senescence response of fibroblasts,[3] and progressively upregulates PAI-1 expression during prolonged culturing in serum.[46]

(4) The serum response profile of lung fibroblasts includes induction of urokinase type plasminogen activator (uPA)[21] and among the activated genes in the poor prognosis wound-like signature are plasminogen and the uPA receptor,[20,22] both of which function downstream of PAI-1 and are antagonized by PAI-1.[50,51]

(5) Mouse models of plasminogen and uPA have inversely correlated wound healing phenotypes as compared to their antagonist PAI-1 or its upstream activator p53. For example, *PAI-1* knockout mice have faster healing wounds, while uPA knockout mice wounds heal slower, and this depends on the proliferation of fibroblasts into the wound.[52,53]

(6) Mice bearing a hyperactive allele of tumor suppressor p53 have premature ageing and reduced wound healing phenotypes.[54]

These observations led us to investigate whether uPA and PAI-1 might be causally involved in the senescence response of fibroblasts to serum in culture and, in turn, whether heterotypic signaling might dictate the proliferative response.

## PAI-1 IN HOMEOSTASIS AND DISEASE

Besides being involved in sepsis and fibrosis, two processes that are intimately tied to chronic infection and inflammation, PAI-1 has a recognized role in atherosclerosis, metabolic disturbances such as obesity and insulin resistance, chronic stress, bone remodelling, asthma, rheumatoid arthritis, glomerulonephritis, metastasis, invasion, angiogenesis, and haemostasis.[50,51,55-58] All these physiological and patho-physiological processes depend on uPA or plasmin activity and are governed by extensive cell-nonautonomous signaling. uPA is a secreted as well as cell surface-bound protease and PAI-1 is the major physiologic regulator of uPA activity; both modulate the extent and duration of extracellular matrix (ECM) remodeling. At the transcriptional level, uPA and PAI-1 are regulated by various growth factors and tumor suppressors, like *c-myc*, *p53*, TGFβ and HIF1α.[59-65] Disturbance of the pericellular proteolytic activity of uPA can result in local ECM degradation by induction of the plasmin protease cascade, and release and activation of matrix metalloproteinases[50] (MMPs). This in turn leads to alteration of local mitogenic signaling, since ECM-degradation results in increased bio-availability of growth factors by liberating molecules like heparin-bound EGF, HGF, bFGF, or IGFs. As a result, there is induction of cell motility and proliferation.[50,55,56,66] The regulation of PAI-1 and uPA signaling and their receptor, urokinase-type plasminogen activator receptor (uPAR), in homeostasis and disease is diverse and complex. For example, normally uPA-PAI-1 complexes bind and activate uPAR, which can

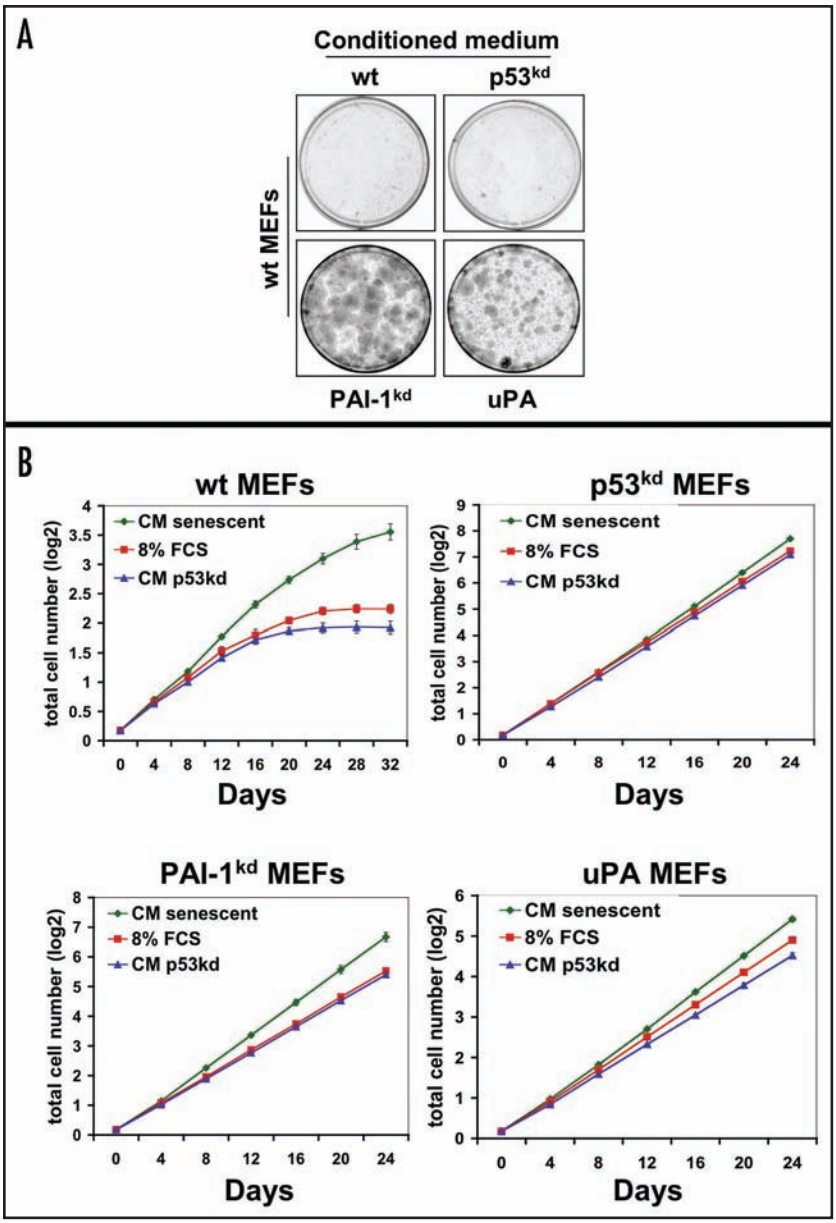


Figure 1. Loss of p53 or PAI-1 expression has a cell-autonomous or noncell-autonomous effect on immortalization. (A) Colony formation assay of late passage wt MEFs cultured with conditioned medium from depicted cell lines. Every 48 hrs media was collected from equal amounts of producer cells and transferred to recipient cells. Starting population of recipient fibroblasts was 50.000 cells per dish. Dishes were stained after two weeks. (B) Growth curves of wild type, $p53^{kd}$, $PAI\text{-}1^{kd}$, or uPA overexpressing MEFs when growing in normal media (8% FCS) or conditioned media (CM) from $p53^{kd}$ or senescent MEFs. Every 48 hrs media was collected from equalized amounts of producer cells and transferred to recipient cells. At is 0 days 150.000 recipient cells were transferred to a 6 cm dish, every four days cells were counted, and 150.000 cells replated. Growth is depicted as cumulative over time. The mean (± sd) per triplicate of three different media additions per genotype is shown.

be blocked by subsequent LDL receptor-related protein mediated binding to uPAR, leading to integration and clearance of the complex and shutdown of uPA signalling.[67] The formation of this quaternary complex therefore regulates proliferation and migration. However, PAI-1 also has uPA independent activity and uPA has PAI-1 independent activity as well. Furthermore, both uPA and PAI-1 also have uPAR independent activity[50,56,68] and, vice versa, the uPAR has both uPA and PAI-1 independent activity.[69] In addition, the physiological response to activity of uPA, PAI-1 or uPAR is cell-type specific. Obviously, aberrant functioning by these molecules is involved in cell growth control, but it is not fully understood why and how.

## PAI-1 AND SENESCENCE

Recently, we have shown that downregulation of *PAI-1* induces immortalization through bypass of senescence in a cell-autonomous fashion in both mouse and human fibroblasts.[70] These data are most readily explained by a model in which suppression of PAI-1 causes activation of uPA, leading to increased bio-availability of growth factors, which in turn stimulate proliferation. Our results support the notion that the observed regulation of PAI-1 and uPA in wound healing and in serum-stimulated fibroblasts are relevant to the proliferative events that take place during these processes. Since PAI-1 and uPA are both extra-cellular proteins, our finding raised at least two new questions. First, does loss of PAI-1 or overexpression of uPA lead to paracrine signaling with growth-stimulatory effects towards adjacent wild type fibroblasts? If so, a possible consequence might be that increased uPA activity in one cell subsequently leads to unresponsiveness to tumor-suppressive p53 signaling in nearby cells. Second and conversely, can senescent cells, which produce elevated PAI-1 levels, induce senescence cell-nonautonomously in other cells? In other words, is senescence-induction contagious?

## SUPPRESSION OF PAI-1 INDUCES SENESCENCE-BYPASS IN A PARACRINE FASHION

We first addressed whether loss of PAI-1 or overexpression of uPA lead to paracrine signaling with growth-stimulatory effects towards wild type fibroblasts. We transferred conditioned medium from immortal PAI-1 knockdown cells ($PAI\text{-}1^{kd}$) or uPA overexpressing MEFs to presenescent wild type MEFs and studied the effect of these conditioned media on the proliferation of the presenescent MEFs. Figure 1A shows that the transfer of conditioned medium from immortal $PAI\text{-}1^{kd}$ (kd: knockdown) or uPA overexpressing MEFs to presenescent wild-type MEFs induced a senescence-bypass in the latter cells. As a control, we used conditioned medium from young wild-type or post-senescent $p53^{kd}$ cells, which did not prevent the onset of the senescence response (Fig. 1A). This suggests that $PAI\text{-}1^{kd}$ or uPA overexpression induces a senescence-bypass that is not strictly cell autonomous. $p53^{kd}$ MEFs have higher amounts of *PAI-1* compared to $PAI\text{-}1^{kd}$ cells[70] and therefore potentially lower uPA activity, and it is possible that this is why $p53^{kd}$ cells were not able to immortalize fibroblasts in a paracrine fashion (Fig. 1A). We note that cell-nonautonomous effects on proliferation caused by p53 have been observed before.[71,72] This brought us to the second question whether senescent cells, which have high *PAI-1* levels and active p53, can induce senescence in young fibroblasts through secretion of PAI-1. Furthermore, since uPA activity is downstream of PAI-1, one might expect uPA overexpressing MEFs to be less sensitive to secreted PAI-1. To address

this, conditioned media from senescent or p53$^{kd}$ MEFs was transferred to wild-type, immortalized p53$^{kd}$ or PAI-1$^{kd}$, or uPA overexpressing MEFs, and subsequently long-term proliferation of the recipient cells was followed. Surprisingly, MEFs of all four genotypes were growth-stimulated by conditioned media of senescent cells (Fig. 1B). This is most likely explained by the findings of Krtolica et al, who showed that senescent fibroblasts secrete mitogenic factors.[12] Apparently, the growth stimulatory effects of the factors secreted by senescent cells are dominant over the growth inhibitory effects of PAI-1 induction. It should be noted however that ectopic expression of PAI-1 in immortal fibroblasts does lead to induction of senescence, consistent with the notion that PAI-1 acts downstream of p53 to block uPA activity.[70] Furthermore, we have found there is a concentration-dependent induction of senescence in immortal cells after administration of recombinant PAI-1, showing that soluble PAI-1 in culture medium is able to induce senescence (Kortlever R and Bernards R, unpublished observations). Thus, the immortalizing effects seen of PAI-1$^{kd}$ are mediated, at least in part, via paracrine signaling. The combined consequences of the autocrine and paracrine effects of knockdown of PAI-1 or overexpression of uPA in ageing fibroblasts are schematically represented in Figure 2. Apparently, in tissue culture conditions senescence is not induced in a cell nonautonomous manner. This does not exclude that in tissue, where cells are more densely packed, high PAI-1 expression might play a role in the induction of senescence in a paracrine fashion.

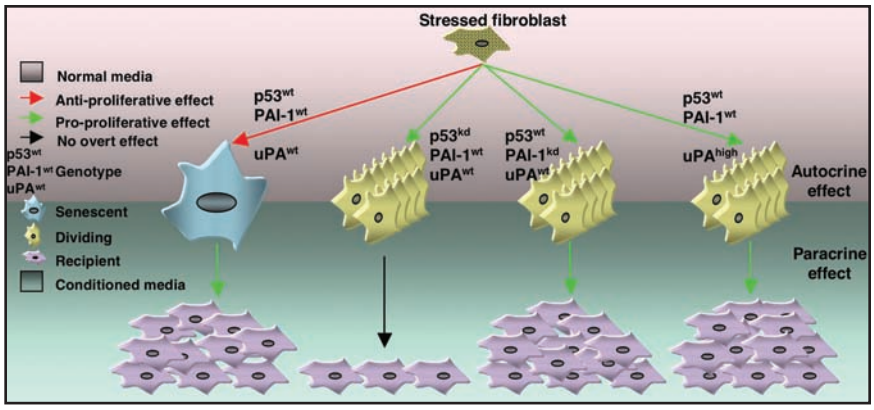


Figure 2. Model of cell autonomous and cell non-autonomous effects on fibroblast immortalization after loss of PAI-1 or uPA overexpression.

## p53 AND PAI-1 REGULATE SENESCENCE VIA SECRETED FACTORS

As was described above, we have found that p53 controls growth factor-dependent proliferation through its secreted target gene *PAI-1*. Ageing fibroblasts, besides upregulating *PAI-1*, *p21$^{CIP1}$* and *p16$^{INK4A}$*, downregulate PI3K-PKB signaling, and the activity of this particular mitogenic signaling pathway might be a central intermediary in the induction of senescence by p53.[8,70] Downregulation of p53 or PAI-1, or overexpression of uPA results in enhanced growth factor signaling through the PI3K-PKB route and stabilization of nuclear cyclin D1. PAI-1 can therefore be regarded an extra-cellular gatekeeper of fibroblast immortalization.[70] These findings also imply that enhanced growth factor signaling via PI3K-PKB deafens cells to the anti-proliferative functions of a group of bona fide tumor suppressors, including p53.

We found that cells become unresponsive to mitogenic signaling by antagonizing uPA through p53-mediated upregulation of PAI-1. Though this shuts down DNA replication of a fibroblast, it does not prevent adjacent cells from being stimulated to proliferate. When the uPA-PAI-1 equilibrium is disturbed to such extent that it favors mitogenic activation, it leaves both producer and recipient cells immortal (see Fig. 2). How can we use this information towards understanding the antagonistic pleiotropy of senescence? It has been suggested by Campisi and colleagues that cells protect themselves during ageing from unlimited proliferation but at the same time such cells stimulate surrounding cells to proliferate.[13] This may be especially important during wound healing, when senescent fibroblasts in a wound stimulate nearby epithelial cells to proliferate and close the wound. Our data are in line with this, and show that two secreted molecules upstream of mitogenic signaling seem to be critically involved in both the pro-proliferative and anti-proliferative aspects of senescence.

## PAI-1, STROMA, AND BREAST CANCER

It is now evident that disturbances of PAI-1-uPA levels can have major implications for proliferation of fibroblasts in both cell-autonomous and cell nonautonomous fashions. Can this insight help us to understand the parallels between wound healing and breast cancer metastasis? As mentioned before, PAI-1 and uPA appear to be central players in senescence and wound healing, and their activation is regulated by inflammation. This might also provide a basis to understand the interactions between stromal tissue and cancer cells. Thus, uPA/PAI-1 deregulation may influence intratumoral heterotypic signaling and alter the tumor microenvironment. Such a model is tempting since: (1) it is becoming increasingly evident that tumors rely on interactions with stromal tissue, of which the principal cells are fibroblasts,[73-75] (2) the transcriptional response of fibroblasts to serum is also seen in the tumors of breast cancer patients having poor prognosis,[22] (3) PAI-1 and uPA are highly expressed by leading edge fibroblasts and myofibroblasts in breast stromal tissue[49,76,77] and (4) PAI-1, uPA and PAR have been prospectively validated as markers of poor prognosis for breast cancer.[78,79] The role and regulation of uPA and PAI-1 during metastasis is complex and sometimes conflicting, though. For example, since PAI-1 antagonizes uPA—which are anti- and pro-proliferative in fibroblasts, respectively—one might expect the knockout mice to have opposing cancer phenotypes. However, absence of host PAI-1 has been shown to reduce tumor burden in tumor transplant or transgenic tumor-induction models.[80,81] Furthermore, both uPA transgenic and uPA knockout mice show reduced metastasis in syngeneic or xenograft mammary tumor models[82-84] and this might be related to hyperactive protease activity or the normal growth promoting role of uPA activity, respectively. In an MMTV-PymT transgenic mouse model of metastasizing breast cancer, PAI-1 knockout in the host has been reported not to lead to metastasis,[81] although in other models it has been shown that in xenograft experiments the PAI-1 levels of the host do seem to be most important in invasion and vascularisation.[80,85] These data appear confusing but probably highlight the complex roles of PAI-1 and uPA in cancer etiology, as it is also not readily explained why both high uPA and high PAI-1 expression are poor-prognosis markers in breast cancer.

## TGFB AND PAI-1

PAI-1 and $p21^{CIP1}$ are not only targets of p53 but also of TGFβ and their activation by TGFβ is dependent on p53.[86] Signaling induced by the cytokine TGFβ can be anti- or pro-proliferative, depending on the genetic context of the recipient cell.[74] As for PAI-1, TGFβ is also involved in fibrosis, wound healing and metastasis and capable of inducing ECM-remodeling by regulating plasmin and MMP activity. Furthermore, TGFβ activity is involved in the conversion of a fibroblast to a myofibroblast,[87] cells that appear to be responsible for PAI-1 secretion at the leading edge of stromal tissue in cancer invasion.[76,77] There is in vivo evidence to suggest that fibroblasts deficient in a TGFβ response can induce the tumorigenic potential of adjacent epithelia.[88] Since PAI-1 is regulated by TGFβ, it will be of interest to test whether PAI-1 has a causal role in the contribution towards epithelial malignancy of TGFβ-unresponsive fibroblasts. There is evidence suggesting that molecules, which themselves are regulated by PAI-1-uPA function, are involved in the pro-tumorigenic cell nonautonomous effect of loss of tumor-suppressive TGFβ activity in fibroblasts.[89] Furthermore, besides that we have shown that enhanced PKB activity seems to protect fibroblasts from anti-proliferative p53 signaling, it has also been reported that hyperactive PKB activity protects cells from a TGFβ-induced $G_1$ arrest.[90,91] In line with this, we have found that constitutively active PI3K-PKB signaling can bypass a TGFβ arrest in Mink Lung cells (Kortlever R, Bernards R, unpublished observations). This implies that loss of p53- or TGFβ-responsiveness can be accomplished by hyper-activation of PI3K-PKB signaling. Apparently, there are analogies between signaling pathways downstream of the uPA-PAI-1 system and TGFβ, whose spatial organization drives cell-cell communication.[70,73,92,93]

## CONCLUSIONS AND OUTLOOK

Our data indicate that both PAI-1 and uPA play critical roles in senescence and that their mode of action is not strictly cell autonomous. Senescence itself is a fundamental biological response to stress and ageing, processes at the heart of cancer. In cancer, the role of cell nonautonomous signaling by inflammatory molecules and fibroblasts is gradually being recognized as a key regulator of disease outcome.[27,29,73,87,93,94] Interestingly, PAI-1 and uPA are also regulated by inflammatory processes. However, it remains to be investigated whether the paracrine effects we find of PAI-1 and uPA towards neighboring fibroblasts also influences proliferation of adjacent epithelial cells. Furthermore, it needs to be addressed whether our findings regarding senescence-induction by PAI-1 can be substantiated in vivo. Nevertheless, it is tempting to speculate that the roles played by PAI-1 and uPA in senescence resembles the roles of these proteins in the interactions between stromal tissue and cancer cells. Stromal tissue defines and creates a niche for metastasizing cells by extensive heterotypic signaling and there is a strong prognostic correlation between the presence of PAI-1 and uPA in stromal fibroblasts and disease outcome. Since both PAI-1 and uPA are molecules involved in cascades of enzymatic processes,[50,51] this might offer a therapeutic window of opportunity to drive cells into senescence. Unfortunately, however, senescent cells themselves stimulate their neighbors to proliferate and therapeutic senescence-induction should perhaps only be considered when the cell nonautonomous effects of senescent fibroblasts can be tamed. Therefore, it will be interesting to see whether the molecules secreted by senescent cells are themselves subject to PAI-1-uPA regulation, and whether PAI-1 and uPA are indeed central players in metastasis. This is worthwhile pursuing, since clinical trials with drugs that inhibit MMP activity, downstream effector-molecules of PAI-1 and uPA activity, have not lived up to their promise yet.[95,96] This might be due to the fact that extracellular signaling in tumors and their metastases is redundant and highly regulated by molecules that have cell-type specific actions.[97-99] Understanding the cell nonautonomous role of PAI-1 and uPA in the induction of senescence might also shed light on the question why the serum response of a fibroblast has such powerful prognostic value for breast cancer.

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