Escaping Growth Arrest in Cancer

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COVER Noon on the Indian Ocean around Mnemba Atoll off Zanzibar, Tanzania, 24 August 1998, 5°49'S 39°23'E.

PAINT STREAK Ensō (円相), or Zen Circle; symbolizing enlightment, strenght, elegance, the universe and the void.

BACK Sunset over the Mediterranian Sea, Sardegna, Italy, 12 September 2006, 41°10'S 9°10'E

Photo's by author.

Escaping Growth Arrest in Cancer

ONTSNAPPEN AAN GROEIREMMING IN KANKER

(Met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 24 juni 2008 des middags te 2.30 uur

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RODERIK MARCO KORTLEVER

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Voor mijn ouders

Hebban olla uogala nestas hagunnan hinase hic enda thu
uuat unbidan uue nu

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If we had never been troubled by celestial and atmospheric phenomena, nor by fears about death, nor by our ignorance of the limits of pains and desires, we should have had no need of natural science.

…

It is impossible for someone to dispel his fears about the most important matters if he doesn't know the nature of the universe but still gives some credence to myths. So without the study of nature there is no enjoyment of pure pleasure.

Epicure, *Principal Doctrines 11 and 12* (341 – 270 BC)

CHAPTER I

General Introduction

Adapted from Cell Cycle 2006 Dec; 5 (23): 2697-2703

General Introduction

Without proliferation no tumor. Mutations in genes implicated in proliferation are most common in cancer. Cells under stress normally have various safeguards against accumulation of DNA damage, but hyperactivity of growth-promoting oncogenes or loss of tumor-suppressors results in continued proliferation in the presence of DNA damage and hence in the fixation of genetic alterations in the tumor cell genome. The bypass of cell cycle checkpoints therefore sets the stage for tumor-progression.

Cancer from a cell autonomous perspective

To overcome tumor-suppression cells have to escape barriers in the cell cycle that normally prohibit uncontrolled proliferation. Malignant behavior of a cell is enforced by mutation, epigenetic or cell-intrinsic changes. The cell cycle is a central intermediary in this since it orchestrates sequences of molecular interactions that collectively entail proper cell division.

The cell cycle

The duplication of a cell into two daughter cells is a tightly controlled process. The cell cycle is divided in two gaps (G1, G2), a DNA replication period (S), and cell division or mitosis (M), ordered in G1, S, G2, and M phases, respectively[1]. Most normal cells in an adult person are quiescent, or non-dividing, and reside in G0. They can be stimulated to re-enter the cell cycle by activation of growth factor signaling pathways that force G0-G1 transition and subsequent bypass of the restriction point in late G1. Once DNA replication is started in S-phase the cell is committed to progression into mitosis independent of mitogens[2]. DNA replication is dependent on cyclins and their associated kinases. D- and E-type cyclins control the G1-S transition, and A- and B-types regulate entry into M phase from G2. D-type cyclins are associated with cyclin dependent kinase CDK4 or CDK6 and E-type cyclins are associated with CDK2, while A- and B-type cyclins are asso-

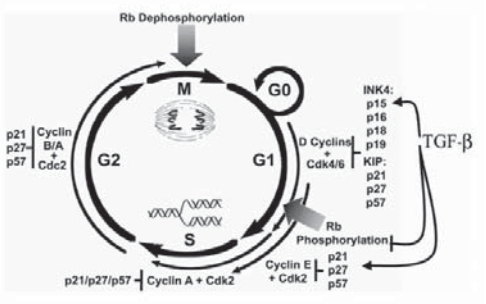

Figure 1 The cell cycle; its activators and inhibitors. See text for details. INK4 is the INK4 family, and KIP is the CIP/KIP family of cell cycle inhibitors.

ciated with cyclin dependent kinase CDK1 or CDK2, or CDK1, respectively ([3]). Cyclin-dependent D- and E-type kinases are stimulated by mitogenic signaling, negatively controlled by inhibitors $p16^{INK4A}$, $p21^{CIP1}$ and $p27^{KIP1}$, and are active in early and late G1, respectively[3,4] (Figure 1).

A mitogenic, or cell growth, stimulus is instigated by growth factors binding to their respective receptor tyrosine kinases at their extra-cellular domain. Binding results in receptor dimerisation and subsequent activation by intracellular autophosphorylation. The resulting association facilitates nucleotide release factors as SOS to interact with its docking proteins Shc and Grb, and this complex mediates activation of mitogenic Ras and PI3K pathways[5]. Ras is a membrane-associated GTPase that can activate multiple downstream effectors as Raf, Ral, and PI3K, which has both poliferative and morphological consequences, depending on the effector and cell type[6-8]. Activation of the kinase signaling pathway most directly regulating proliferation, Ras-Raf-Mek-Erk, has several effects. The first results in recruitment of the prototypical Ets/Ap-1 complexes[9] and production of growth promoters as *cyclin D1*. Secondly, it activates serum response elements or serum response factors leading to induction of immediate-early genes as Egr1, or proto-oncogenes *c-myc* and *c-fos*[8,10]. Lastly, kinase activity downstream of Ras signaling results in association of cyclin D1-CDK4 complexes and enhances their stabilization[4,11].

Membrane-associated PI3K on the other hand induces the transition of phosphatidylinositol 4,5-biphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3) by phosphorylation, which activates PDK1 and subsequently PKB. This PI3K-PKB kinase cascade is then responsible for inactivation by phosphorylation of certain targets normally suppressing survival and proliferation, like GSK3β, $p27^{KIP1}$, and various Forkhead transcription factors[12].

Ultimately, both Ras and PI3K signaling pathways converge on cyclin D1 ([13]). D-type cyclins are short-lived proteins and their synthesis and assembly with CDK4 or CDK6 is dependent on mitogenic signaling[14,15]. Cyclin D1-CDK4/6 complexes phosphorylate the retinoblastoma protein pRb in early G1, partially de-repressing the E2F family of transcription factors by alleviating pRb inhibition[4]. Production of E2F target *Cyclin E* and subsequent Cyclin E/CDK2 activity will lead to a more extensive pRb phosphorylation in late G1, complete release of E2Fs, and the onset of S-phase.

pRB is a key switch controlling growth-factor dependent proliferation[20-23]. Constitutive growth factor signaling triggers growth-suppressive activity through two pathways, converging on pRb. Hyperactive Ras induces both $p16^{INK4A}$ and $p19^{ARF}$-p53 ($p14^{ARF}$-p53 in humans), and supra-physiological PI3K-PKB signaling triggers p53 ([16,17]). $p21^{CIP1}$ is a potent downstream target of p53 and inhibits cyclin D- and E-dependent kinase activity, while $p16^{INK4A}$ inhibits Cyclin D1/CDK4 specifically[4]. Conversely, absence of mitogens reduces *Cyclin D1* transcription and activation of Forkhead transcription factors. $p27^{KIP1}$ is thus induced, restricting inhibition of pRb[18,19]. Collectively, mitogenic signaling by activation of Cyclin D1/CDK4/6 and Cyclin E/CDK2 cyclin-dependent kinase complexes balances with inhibition by $p16^{INK4A}$, $p27^{KIP1}$ and $p21^{CIP1}$ on the level of regulation of the pRb G1 cell cycle restriction point.

Uncontroled intracellular signaling

Since, as outlined above, p53 and pRb are sensitive to growth factor signaling, it is not surprising that a multitude of various Ras and PI3K signaling-related oncogenic mutations are found in cancer and other disease[24,25]. Oncogenes are activated as a result of subtle intragenic

mutations that regulate the activity of the gene product, gene amplifications, or chromosomal translocations, permitting abnormally high levels of activity[26]. For many different families of receptor tyrosine kinases activating mutations or overproduction of their ligand is observed in cancer[27-29]. Besides this, activating Ras mutations are found in a high percentage in a wide spectrum of cancers[30]. BRAF, a downstream effecter of Ras, is most commonly mutated in human cancer at only a single residue, $V \rightarrow G^{599}$, constitutively activating the enzyme[31]. Late stage colorectal tumors have a high frequency of mutation in p110α, the catalytic subunit of PI3K ([32]). Conversely, loss of function mutations of PTEN, an inhibitor of PI3K, are associated with breast, colon, and prostate tumorigenesis[33]. Significantly, in breast cancer, the combined mutation status of PI3K and PTEN and hence their pathway activity may have a predictive value regarding Trastuzumab treatment[34]. Enhanced activity of PKB, a downstream mediator of PI3K, by mutation or direct regulation is associated with many types of cancer[35,36]. Next to enhanced Cyclin D1 expression in 50% of breast cancer, amplification of *Cyclin D1* or induction of *Cyclin D1* transcription is prevalent in for example breast, head and neck squamous cell, lymphoid or parathyphoid cancer[37,38]. In short, hyper-activation of mitogenic signaling pathways is an integral part of cancer growth.

As uncontrolled proliferation is a hallmark of cancer, much attention has been focused on escaping tumor suppression. Central herein are the p53 and pRb tumor suppressors. Their value was first appreciated when they were found to be specifically inhibited by binding cancer associated DNA viruses. Simian Virus 40 uses Large T to target both p53 and pRb, while Human Papilloma Viruses (HPV) use E7 and E6, respectively[22,39]. The viruses thus actively promote entry into cell cycle of the infected host cells to favor viral DNA replication. The loss of function of p53 and pRb can be brought about in various ways: through epigenetic silencing, inhibition (e.g. by blocking by direct binding, or of its expression), or mutation[40,41]. Loss of p53 function will be discussed in more detail further below. pRb is member of a family of so-called 'pocket proteins': pRb, p107 and p130. The term 'pocket proteins' is derived from the conserved binding region through which they bind viral oncoproteins and cellular factors such as the E2F family of transcription factors[42]. pRb was first found to be mutated in hereditary retinoblastoma, a sporadic pediatric neoplasm arising from retinal cells[43,44], and it is suspected that numerous cancers, if not all, show a deregulated pathway[38,45]. An upstream regulator of pRb, $p16^{INK4A}$, is epigenetically silenced or mutated in lung tumors. Inactivation of pRb or $p16^{INK4A}$ may be cell-type specific though, as 80% of small cell lung tumors harbor pRb mutations, while $p16^{INK4A}$ loss occurs in the majority of non-small cell lung cancer[4]. Functional inactivation of pRb by viral oncoprotein binding is observed in many neoplasias as cervical cancers, mesothelioma and AIDS related Burkitt's lymphoma[46].

Apparently, uncontrolled proliferation by bypass of growth-inhibition can be brought about by hyper-activation of mitogenic signaling or by mutation or blocking of their downstream negatively regulated targets, in the latter case rendering the cells independent of growth factors.

Cancer from a cell non-autonomous perspective

In cancer the molecular cross-talk between cell types is disrupted, leading to invasion and migration. The innate homeostatic program appears hijacked and consequently there is a pathophysiological state.

Heterogeneity

The idea of tumor-heterogeneity has come a long way, and it emerged from pathological analysis. Seminal observations on cancer development came in the late 19th century from both Virchow and De Morgan, who found inflammatory processes and spread to other tissue, respectively. Virchow proposed that the immune system may be tightly connected to cancer progression, and in the 1980's through novel in vitro assays and culturing techniques a parallel between wound-healing and cancer progression was observed in controlled experimental settings[47-50]. Chicken embryo's with locally injected cells transformed by Rous Sarcoma Virus showed no growth induction in normal tissue, but malignant spread at the site of inflamed fresh wounds, next to growth at the site of injection[49]. The idea that tumors may be more than just clonal expansion did not adhere easily though[51]. Only recently has it been appreciated that the erratic behavior of tumors may indeed be highly dependent on heterotypic signaling.

Perturbations of the inflammatory pathway is strongly associated with tumor progression. Tumors are composed of innate and adaptive immune cells, epithelial cells, fibroblasts, lymphocytes and other mesenchymal cell types[52-57]. Each cell type has exquisite influences on tumor progression. Gene-expression profiling of breast cancer uncovered that genetic programming coupled to patient survival is heavily influenced by inflammatory processes[58]. Further, infiltrates of macrophages in human breast cancer or mast cells in pulmonary adenocarcinoma have been associated with bad prognosis[59,60]. In a mouse model of breast cancer, ablation of the gene encoding a macrophage growth factor, colony-stimulating factor (CSF-1), reduced the rate of tumor progression and establishment of metastases[61]. A similar observation in a xenograft model linked mutation of CSF-1 to reduced invasion of host-derived tumor-associated macrophages[62]. The critical proliferation-control protein p53 interacts with inflammatory processes of juxtaposed cells. Through suppression of NF-κB p53 controls the innate immune response, buffering inflammation in mice[63]. Besides this, there's also other heterogeneous processes in the tumor-stroma. Mutations in pRb and subsequent activation of p53 signaling in prostatic intraepithelial neoplasia can lead to evolution of paracrine selective pressure to mutate p53 in adjacent stromal fibroblasts[64]. In a co-implantation tumor xenograft model for studying stromal mesenchymal tissue, overexpression of the chemokine stromal derived factor-1 in specific breast cancer associated patient fibroblasts can enhance tumor formation when co-injected with breast cancer epithelial cells in a mouse[65]. Further, stromal fibroblasts isolated from human prostate carcinomas increase tumor formation when compared to normal fibroblasts[66]. Co-culture and *in vivo* experiments show that normal stromal tissue can suppress tumorigenicity, but that tumor-associated or irradiated stromal tissue supports oncogenic growth of recipient non-tumorigenic cells[67,68].

Another major proliferation-affecting pathway altered in many cancers is TGFβ. Ablation of tumor-suppressive TGFβ activity by deletion of the TGFβ receptor type II (TGFβRII) in prostate fibroblasts in mice leads to intraepithelial neoplasia and squamous cell carcinoma of the forestomach, both associated with an increase in stromal cells, by induced growth factor signaling towards the epithelium from the fibroblasts[53,69]. Additionally, conditional knockout mice for TGFβRII in stratified epithelia rapidly developed spontaneous squamous cell carcinoma in the anogenital region with high penetrance[70]. Though there was local inflammation, wounding was not sufficient for spontaneous carcinogenesis. Another mechanism suspected to be instructive for evasion and which involves TGFβ and immune activation is epithelial to

mesenchymal transition (EMT) ([71]). EMT may be responsible for trans differentiation of tumor epithelial cells to myoepithelial cells. The altered cells may have more havoc-raging capabilities and lead to metastasis, by influence of embryogenic factors such as the transcription factors Slug, Snail, Twist and Goosecoid[72-75].

Thus, slowly we now start to understand the identity of some of the cross-talk between tumor cells and the environment. Genes involved in tumor-initiation seem to be closely linked to heterotypic signaling pathways promoting tumor-progression. Slowly a picture of tumor-progression is emerging: intrinsic epigenetic changes or mutations in tumor cells selectively forces tumor progression by activating the environment. As a result leukocytes, lymphocytes, macrophages, and fibroblasts can alter the tumor-associated microenvironment by extensive cell non-autonomous signaling, and thus promote a continuous bypass of growth- and migration-inhibiting processes[52-57]. Collaboration between the attracted immune-system and the mesenchyme results in activation of fibroblast tissue in stroma, leading to so-called 'cancer-associated fibroblasts' or CAFs, possibly paving the way for localized escape of homeostasis by shifting the balance towards proliferation, angiogenesis, and invasion[76]. The observed phenotypic characteristics of cancer are therefore ultimately determined not only by the genetic blueprint of the tumor-initiating cells, but also that of the recruited micro- and macro-environment.

Extra-cellular signaling

Instructive information to the cell originates from the extra-cellular matrix (ECM) environment. Its architecture defines a scaffold for cell-cell communication. The effects of the ECM are predominantly mediated by Integrins, a family of cell surface receptors that attach cells to the matrix and mediate mechanical and chemical signals. These signals regulate the activities of cytoplasmic kinases, growth factor receptors and ion channels, and control the organization of the intracellular actin cytoskeleton[77]. Both *in vitro* and *in vivo* migration and invasion studies have shown that extra-cellular signaling-pathways are essential for establishing the architecture distinguishing normal from tumor-tissue[78].

Invasive behavior is dependent on pathways stimulating angiogenesis, altering inflammation responses leading to resistance to apoptosis, or ones clearly influencing morphology and local extra-cellular enzymatic activity[78]. ECM-associated factors direct intracellular signaling pathways by interacting with their trans-membrane receptors or by morphological cues. As a consequence, tissue-specific gene expression and phenotypes are established. The natural mesh of structural proteins harbors a pool of pro-enzymes regulated by and regulating environmental cues. The intricate interplay of soluble and insoluble cell-type specific factors is highly dynamic and resolves around cascades of (ant)agonizing proteolytic activities exemplified by matrix metalloproteinases (MMPs) and serine protease uPA ([79-81]). These enzymes themselves also function as growth factors or their upstream regulators since they dominate scaffolding and release of ECM-associated growth factors[82]. Most MMPs in a tumor are expressed by host stromal and inflammatory cells in response to factors released by the tumor cells[83]. Just as uPA and its antagonist plasminogen activator inhibitor 1 (PAI-1), MMPs and their inhibitors TIMPs can have redundant or even opposite roles in tumor progression[82,83]. MMP and uPA activity are not exclusively in parallel but also in functionally overlapping pathways[84]. Homeostasis is thus ultimately dependent on a complicated cross-talk between growth factors and their inhibitors originating from various cell types, bound by the ECM.

The timely onset of proliferation and differentiation is tightly regulated by endothelial cell production of angiogenic factors as VEGF or PAI-1, growth factors as IGF, EGF, LPA, HGF, or bFGF by fibroblasts, or cytokines and chemokines as interleukins, TGFβ or TNFα by fibroblasts. Recruited inflammatory cells are kept in check by their inhibitors or inhibitory pathways[54,56,85,86].

Growth promoters and -inhibitors

We are currently aware of a number of key signaling pathways that transmit growth and differentiation signals to the nucleus. Not all components of these pathways are equally important in cancer. A few of the key players, which are also the subject of this thesis, are discussed in more detail below:

p53

The p53 tumor suppressor gene encodes a transcription factor that plays a seminal role in the response of mammalian cells to physiological and environmental stress, and is the main mediator of cell cycle arrest and apoptosis after genotoxic insult[87-90]. Full length human p53 encodes a 393-amino acid transcription factor composed of N-terminal transactivation domains that interact with co-activators or co-repressors, a sequence specific DNA-binding domain, a tetramerization domain and an extreme carboxy-terminal regulatory domain. In normal unstressed cells p53 levels are low and p53 is kept inactive. In stressed cells, p53 is stabilized, accumulates, and is post-translationaly modified[91]. p53 transcriptional targets, as Mdm2 (HDM2 in man), Pirh2 and COP1, function as ubiquitin ligases that function to facilitate p53 degradation via the ubiquitin-proteasome pathway in a feedback response[89,92]. Mdm2's ability to ubiquitinate p53 results in shuttling of p53 from the nucleus into the cytoplasm, interrupting p53 transcriptional activity[93]. Mdm2 is in a linear pathway with $p19^{ARF}$, an upstream activator of p53 activity, since $p19^{ARF}$ activation results in binding and recruitment of Mdm2 to nucleoli, leading to loss of its inhibition of p53 ([89,93,94]). In response to stress stimuli p53 activation mediates apoptosis induction, growth arrest or senescence. The latter process will be discussed in more detail further below. Perturbations such as DNA damage, mitogen withdrawal, or ectopic expression of proto-oncogene *c-myc*, E2F1, adenovirus E1A, SV40 Large T or HPV E7 can arouse p53-dependent apoptosis[89,95,96]. Hyperactive oncogenes as *Ras*, oxygen, excessive growth factor signaling, DNA damage, or cytokine stress can induce G1 arrest, or senescence[89,95,97].

The physiological outcome of p53 activation is determined by specific phosphorylation or acetylation events[91]. Phosphorylation of human p53 at residue Ser15 (Ser18 in mice) is important for recruiting co-activators to target promoters and active p53-responses to DNA damage in a promoter- and cell-type specific manner. In mice phosphorylation of Ser18 and Ser23 are critical to activate p53-dependent apoptosis after DNA damage and suppress tumorigenesis. Human p53 phosphorylation of Ser 46 and Ser33 may be involved in UV-dependent apoptosis induction. Key targets implicated in apoptosis include Bax, FasL, Puma or Noxa, while targets as $p21^{CIP1}$ or GADD45 are suspected to be involved in a p53-dependent cell cycle arrest[89] (Figure 2).

An estimated 50% of cancers harbor mutations in p53 ([98]), therefore it is now recognized as being the single most mutated gene in cancer. A kaleidoscope of mutations have been registered for p53 ([99,100]). Cancer prevention by p53 is viewed as a result of its guarding of genomic instability through elimination of genetically damaged cells by its growth-suppressive and apoptotic functions. Shortly after the first observations that viral oncogenes could

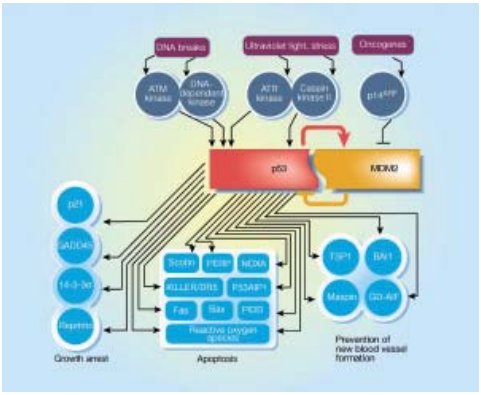

Figure 2 The p53 network.

Activation of the network (by stresses such as DNA damage, ultraviolet light and oncogenes) stimulates enzymatic activities that modify p53 and its negative regulator, MDM2. This results in increased levels of activated p53 protein. The expression of several target genes is then activated by binding of the activated p53 to their regulatory regions. These genes are involved in processes that slow down the development of tumors. For example, some genes inhibit cell-cycle progression or the development of blood vessels to feed a growing tumor; others increase cell death (apoptosis). A negative feedback loop between MDM2 and p53 restrains this network. Many other components of this network, not shown here, have been identified. Similarly, p53 activation results in a variety of other effects, including the maintenance of genetic stability, induction of cellular differentiation, and production of extra-cellular matrix, cytoskeleton and secreted proteins. The components of the network, and its inputs and outputs, vary according to cell type. p53 is a highly connected 'node' in this network. It is therefore unsurprising that the loss of p53 function is so damaging, and that such loss occurs in nearly all human cancers. (© Vogelstein, Lane & Levine, 2000)

block p53 function and that p53 suppresses transformation, mutations of p53 were found in colon tumors and it was linked to the familial Li-Fraumeni cancer susceptibility syndrome[101]. Though the half-life of p53 is low in normal cells and increases after activation, many cancers contain high p53 levels. This is sometimes used as a prognostic marker, though the validity of this is disputed when subjected to meta-analysis[102,103]. That high p53 may be a sign of bad prognosis sounds contradictory, but the conformation of a mutated non-functional p53 is different from the normal form, as exemplified by an increase in the half-life from minutes to hours[104]. Indeed, a missense mutation in lung cancer is an independent, unfavorable prognostic factor[105]. Additionally, Mdm2, the upstream regulator of p53, was recently found to have a single-nucleotide polymorphism in its promoter (SNP309), correlating with lower age of onset of tumors and an increase in the occurrence of multiple primary tumors in Li-Fraumeni syndrome, and accelerate the development of sporadic adult soft tissue sarcoma[106]. However, this polymorphism is also observed in other cancers[107], yet it is not necessarily is linked to prognosis[108], while this does count for amplification or induced expression of Mdm2[109,110].

Transgenic and knockout p53 mouse models have yielded detailed information on the role of p53 in tumorigenesis[111-113]. The vast majority of $p53^{-/-}$ mice die of neoplastic diseases, predominantly lymphomas, before reaching 6 months of age. Through creating tissue- or oncogene-specific models p53's involvement in cancer has been unequivocally established. Interestingly, sophisticated timing- and tissue-specific 'on-off' models have provided us the idea that reactivating p53 in a tumor may be worthwhile pursuing as a therapy as this results in massive clearance[114,115].

Clearly, p53 is at the crossroads of a wide range of stimuli and the cell-specific response to them. Furthermore, on the level of transcription-regulation, p53 can converge with other stress-related pathway's, like TGFβ signaling[116].

TGFβ

The cytokine transforming growth factor-β (TGFβ) controls diverse processes, including cell proliferation, differentiation and stress

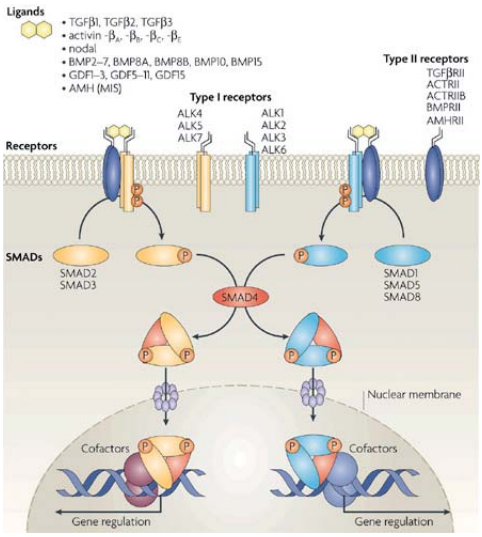

Figure 3 Core signaling in the mammalian TGFβ–SMAD pathways.

Binding of ligands to type II receptors and recruitment of type I receptors involves high combinatorial complexity. Traditionally, ligands have been split into two groups: SMAD2- and SMAD3-activating TGFβs, activins and nodal on the one hand, and SMAD1, SMAD5 and SMAD8-activating BMPs, GDFs and AMH on the other hand. Note that this concept does not accurately reflect reality. The pathway only splits into two distinct branches downstream of type I receptors: ALK4, ALK5 and ALK7 specifically phosphorylate SMAD2 and SMAD3, whereas ALK1, ALK2, ALK3 and ALK6 specifically phosphorylate SMAD1, SMAD5 and SMAD8. Complex formation of the phosphorylated receptor-regulated SMADs with SMAD4 causes nuclear accumulation of active SMAD complexes, which directly regulate gene transcription in conjunction with transcription factors, chromatin-remodelling complexes and histone-modifying enzymes. ACTR, activin receptors; ALK, activin receptor-like kinase; AMH, anti-Muellerian hormone; BMPs, bone morphogenetic proteins; GDFs, growth and differentiation factors; TGFβs, transforming growth factor β ligands. (© Schmierer and Hill, 2007)

responses[23,117-119]. TGFβ has three isoforms, of which TGFβ-1 is the most commonly implicated in tumorigenesis. Cell surface proteoglycans enhance TGFβ binding to TGFβRII. Ligand binding to TGFβRII results in phosphorylation and activation of TGFβRI. Receptor-associated SMAD2 and SMAD3 proteins are phosphorylated and released from the membrane complex. They then bind in a heterotrimeric complex with co-SMAD SMAD4 and translocate to the nucleus[120,121]. The complexes directly or indirectly bind to TGFβ-responsive promoters, and cooperate with general transcription factors to regulate a wide range of target genes[121,122] (Figure 3). Next to a canonical SMAD2/3 dependent pathway, there is an alternative SMAD1/5/8 pathway, used by bone morphogenetic protein (BMP) and involved in embryogenesis. This latter pathway will not be further discussed here. TGFβ target genes are involved in development, wound healing, inflammation and proliferation. In cancer TGFβ induces genes associated with invasion and cell motility, including increased expression of secreted ECM-associated proteins such as plasminogen activator inhibitor-1 (*PAI-1*) and multiple matrix metalloproteases[121,123,124] (MMPs). TGFβ has pleiotropic actions with important consequences. TGFβ is known to inhibit growth in early tumorigenesis, but is associated with stimulating aberrant behavior in later stages[23,123,125,126]. The growth-suppressing or cytostatic effect is mediated by the induction of cyclin dependent kinase (CDK) inhibitors $p21^{CIP1}$ and $p15^{INK4b}$ and suppression of proto-oncogene *c-myc*, and is dependent on retinoblastoma (pRb) function[127-132]. In short, by suppressing growth factor signaling dependent CDK activity TGFβ pathway activation leads to a G1 arrest.

TGFβ pathway mutations include ligand and receptor mutation, deletion or amplification[117,133]. Mutated or altered expression of

SMAD family members is observed in cancer. Mutations of the TGFβ receptors have been found in ovarian, breast, and pancreatic cancers, as well as in lymphomas. The mechanisms behind this are difficult to interpret: sometimes high expression of the receptors, while in other cases loss of TGFβ signaling is the culprit. Low levels of *TGFβRII* gene expression correlates with an increased risk of breast cancer[134]. In colon, mutations in *DPC4*, the gene coding for SMAD4, are often noted[135]. Conversely, over-expression of TGFβRII in the stromal compartment of breast cancer correlates with poor prognosis[136], suggesting cell-autonomous signaling by TGFβ may be responsible for neoplastic transformation of adjacent epithelial cells. In prostate cancer TGFβ up-regulation is associated with angiogenesis, metastasis, and poor prognosis[137]. Obviously, misregulation of TGFβ has a significant impact on tumor progression and patient prognosis, but since the sensitivity depends on the specific tissue- and genetic background, understanding TGFβ signaling is a daunting task.

In humanized mouse models, many cancer-related studies on TGFβ have shown its involvement in delaying early tumorigenesis or illustrating its contributions to malignant behavior at later stages. Since TGFβ has both pro- and anti-proliferative targets, this further suggests that the genetic predisposition of recipient cells determines whether the response to TGFβ is growth or growth arrest. Next to mutation in *TGFβRII* or *SMAD4*, alteration of $p15^{INK4b}$, $p21^{CIP1}$ or *c-myc* levels can shift the cytostatic response towards a growth promoting role of TGFβ[117,118,121,125,127,138]. Interestingly, with regard to TGFβ's relation with the haematopoietic system, it has been found that loss of $p15^{INK4b}$ function by methylation of its promoter relates to acute leukemia and myeloproliferative disorder[139,140], and that in mice amplification of *N-myc* results in acute myeloid leukemia[141]. Though mutations of TGFβ pathway components are common, they cannot account for the majority of cases where bypass of tumor-suppressive TGFβ occurs in the presence of apparently normal TGFβ receptor and SMAD function. Ultimately, TGFβ appears to be a switch connecting tumor-initiation and tumor progression. To better understand how during tumor progression cells evade the growth-limiting response to TGFβ, we need to study its regulation of the G1 cell cycle checkpoint.

PAI-1

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 remodeling, asthma, rheumatoid arthritis, glomerulonephritis, metastasis, invasion, angiogenesis, and haemostasis[142-147]. All these physiological and patho-physiological processes depend on uPA or plasmin activity and are governed by extensive cell-non-autonomous 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 extra-cellular 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 ([148-154]). The stoichiometry of uPA-PAI-1 complexes has its influence on matrix proteins as vitronectin, laminin, and fibronectin[78,155]. 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[145] (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[142,143,145,156]. A lot of research has been focusing on the role of PAI-1 in the blood-system. Mice knockout for *PAI-1* have no overt phenotype, but many (patho) physiological condition-studies have confirmed PAI-1 has a functional role in angiogenesis and disease progression, and seems to be involved in many diseases where there's specific disturbance of proper haemostasis[142,145,147,157-159].

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 be blocked by subsequent LDL receptor-related protein mediated binding to uPAR, leading to integration and clearance of the complex and shutdown of uPA signaling[160]. This quaternary complex formation 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[143,145,161] and, *vice versa*, the uPAR has both uPA and PAI-1 independent activity[162]. In addition, the physiological response to activity of uPA, PAI-1 or uPAR is cell-type specific. Interestingly, recently it was found that PAI-1 is involved in oncogene-induced haemostasis. In a mouse model targeting the *MET* oncogene in liver it was observed that slowly progressing hepatocarcinogenesis was accompanied by blood hypercoagulation and subsequent fatal internal haemorrhages. This pathogenic response was dependent on transcriptional induction of *PAI-1* and *COX2* and they both supported the thrombohaemorrhagic phenotype. Apparently, next to being an active player in invasion and migration, PAI-1 is instrumental in the link between blood coagulation and cancer[144]. Moreover, via this mechanism PAI-1 is suspected to have a functional role in early cancer development[163]. Additionally, the ligand for the MET receptor (which is normally expressed both in stem cells and cancer cells, and a key regulator of invasive growth), or scatter factor/hepatocyte growth factor (HGF), when produced by TGFβ unresponsive fibroblasts in the prostate, can induce cell-nonautonomous adenocarcinoma formation in adjacent epithelial tissue[164,165].

Obviously, aberrant functioning by PAI-1 may very well be directly involved in cell growth control, but it is not fully understood why and how.

Studying G1 regulation

At the G1 cell cycle phase a flood of signals that influence cell fate have to be integrated. Much research has focused on upstream regulators as p53 and TGFβ, since they determine induction of *bona fide* growth-inhibiting processes: senescence or cytostasis. Escape of proliferation-arrest and subsequent transformation of the tumor cells and their environment is key to tumorigenesis, and therefore an in-depth understanding of p53- and TGFβ-signaling is required. Conveniently, the physiological response of cells to stressful culturing may provide a tool in hunting for answers.

Senescence

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 G1 cell cycle arrest, remain viable. This phenomenon was first recognized by Hayflick in 1961 ([166]) 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-sup-

pressive effects of the pRb and p53 pathways[167]. Therefore, new insights into the function of many fundamentally critical cancer-related genes have been obtained by studying replicative senescence in fibroblasts[4,168]. This may be of direct clinical value, as recently it was confirmed that both in mouse and human tumorigenesis senescence is a *bona fide* tumor suppressive mechanism *in vivo*[17,169-171]. 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 ([23,167,172,173]). But what happens downstream of p53? Second, how can senescent cells, which themselves do not proliferate, stimulate proliferation of adjacent cells[174,175]? Why do senescent cells have these two faces? We here reflect on possible implications of these observations for our understanding of fibroblast behavior, wound healing, and metastasis, and will elaborate on our findings connected to these questions in the Discussion (see Chapter 5).

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 G1 phase of the cell cycle[4,176]. The senescent cells have an enlarged morphology and an absence of DNA synthesis. This senescence response reflects a fail-safe mechanism that acts to protect cells from aberrant growth and oncogenic transformation *in vivo*[172,177]. The growth arrest is accompanied by a gradual up-regulation of CDK inhibitors $p21^{CIP1}$ and $p16^{INK4A}$, cell cycle inhibitor $p19^{ARF}$, and p53. $p16^{INK4A}$ works upstream of pRb to induce a cell cycle arrest, and $p19^{ARF}$ activates p53 by inhibiting Mdm2. 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[4,23,168]. In human cells the tumor-suppressor $p16^{INK4A}$ and telomere-erosion also play roles in senescence-induction[172,178]. The tumor-suppressive functions of p53 and pRb can 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[167].

In normal fibroblasts, proliferation is induced by extra-cellular growth factors. They stimulate mitogenic signaling leading to activation of cyclin-dependent kinases (CDKs), which in turn inactivate pRb's growth-inhibitory activity. Such mitogenic signaling is essential for cells to pass the pRb-controlled G1 cell cycle checkpoint, which is often deregulated in cancer[4,23]. 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[179]. 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[180] and a dominant negative mutant of E2F (E2F-DB) immortalizes MEFs in the presence of an activated $p19^{ARF}$-p53 pathway, making E2F transcription factors essential downstream components in the G1 arrest[181]. 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, an integration of three physiological responses of fibroblasts is dis-

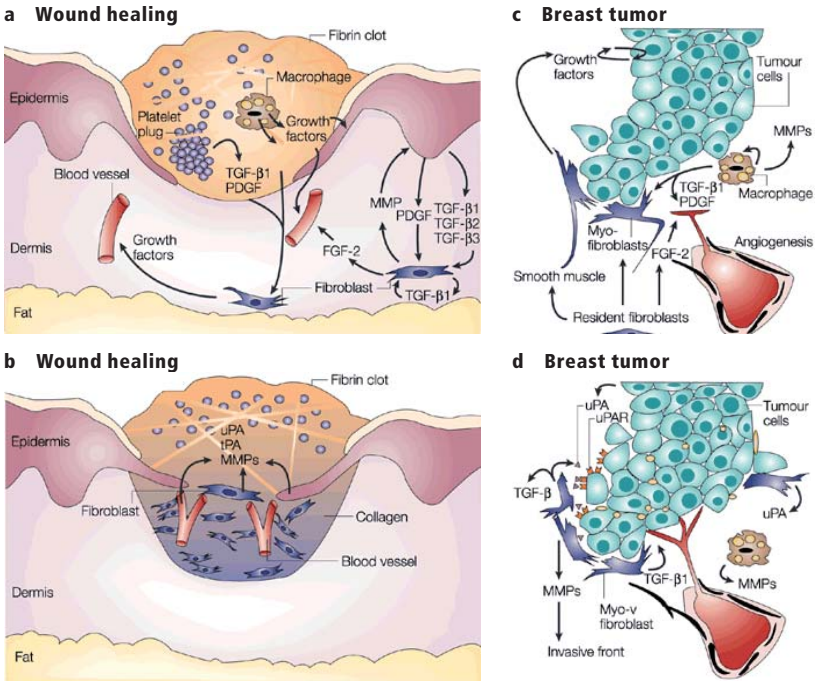

Figure 4 Comparison between wound healing and tumor development.

Wound healing and tumor development are dynamic, progressive processes that involve the interaction of several tissue types, and comparison of the two reveals many mechanistic similarities.

a. Immediate reaction to wounding. Tissue injury leads to activation of platelets that form a haemostatic plug and also release vasoactive mediators to increase vascular permeability and to enable the influx of serum fibrinogen to generate the fibrin clot. Platelets produce chemotactic factors, including transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF). These factors initiate the formation of granulation tissue by activating fibroblasts to produce matrix metalloproteinases (MMPs) and a number of growth factors, such as fibroblast growth factor-2 (bFGF/FGF-2). These factors degrade dermal extracellular matrix, stimulate infiltration of macrophages and promote the development of new blood vessels. These interactions are potentiated by reciprocal signaling between the epidermis and dermal fibroblasts through growth factors,MMPs, and members of the TGF-β family.

b. Reformation of the epithelial sheet. The complex reaction to wounding reduces epithelial adhesiveness and increases epithelial-cell mobility to re-form an intact sheet of tissue over the wound. Production of MMPs and proteolytic enzymes such as uroplasminogen activator (urokinase-type plasminogen activator; uPA) and tissue plasminogen activator (tPA) facilitates this re-epithelialization. Blood vessels can then enter the fibrin clot as epidermal cells resurface the wound. The lateral migration of the epidermal cells is followed by a reversion to the normal, non-motile phenotype, including regeneration of a basement membrane and resynthesis of hemidesmozomes. Following re-epithelialization, a new basement membrane is produced and many of the fibroblasts take on a myofibroblast phenotype to facilitate wound contraction.

c. Reciprocal activation mechanisms in early tumors. Building on a rich, but inconclusive, literature spanning nearly a century, Dvorak proposed that tumors activate some of the normal wound-healing responses. Although developing tumors do not disrupt the vascular tissue in the same way as in wounding, many of the processes occur in parallel. Tumor cells produce many of the same growth factors that activate the adjacent stromal tissues in wounding or fibrosis. Activated fibroblasts and infiltrating im-

cussed: 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[182-184]. Micro-array analysis of fibroblast proliferation induced by serum-addition shows a gene-signature resembling that of an activated fresh wound[182], and this wound-like profile is a poor prognosis marker for multiple types of adenocarcinomas[58,184]. Interestingly, as mentioned earlier, it was noted already some time ago that both inflammation and wound-healing may be linked to cancer[50,185,186]. Over the past years these links have been substantiated by showing that molecules known to play a role in inflammation and wound healing are also causally involved in cancer[55,85,165,185,187,188] (Figure 4). 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[189,190]. 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[189,191,192].

There are several similarities in the molecular events that take place in fibroblasts in response to serum and during aging, wound healing, and the induction of replicative senescence. For example: (i) mouse models show that healing wounds depend on fibroblasts for closure[190,193], (ii) chronic human wounds contain fibroblasts with diminished or absent replicative potential, and this seems to be telomere-erosion independent[194-196] (iii) senescence *in vitro* is regarded premature aging of cells[166,197], (iv) fibroblasts of elderly people have shorter replicative potential when cultured, (v) increased age is correlated with poor prospects for healing of a wound[198], (vi) there is presence of senescent cells in age-related pathologies[199], and (vii) the number of senescent fibroblasts increases exponentially in the skin of aging primates[200]. So, which genes may 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?

Cytostasis

Gene-ablation and TGFβ pathway mutation studies have firmly established its causal role in tissue growth and morphogenesis. During embryonic development, angiogenesis and vasculogenesis are dependent on TGFβ. However, though early in embryogenesis TGFβ has no growth-inhibiting effect, as tissue matures many cell types gain the ability to respond with a growth effect or apoptosis[201,202]. This obviously supports the notion that the variable nature of a recipient cell determines the physiological response to the cytokine. The canonical TGFβ SMAD-dependent pathway is the central mode of action, but the local microenvironment determines the cellular response[23,123,125,126]. In the later stages of tumor development, TGFβ is a tumor promoting agent rather than a negative

mune cells (macrophage) secrete MMPs and cytokines such as TGF-β, FGF-2, and PDGF. These factors potentiate tumor growth, stimulate angiogenesis, and induce fibroblasts to undergo differentiation into myofibroblasts and into smooth muscle.

d. Expression of proteases at the invasive front. Tumor cells, myofibroblasts and activated macrophages increase production of MMPs and uPA at the invasive front to stimulate angiogenesis and proliferation. Production of TGF-β also promotes tumor growth. uPAR, uroplasminogen receptor. (© Bissell and Radisky, 2001)

regulator of growth. As tumors progress from adenoma to an invasive carcinoma, the cytostatic response to TGFβ is lost and subsequent selection facilitates the acquisition of additional oncogenic mutations, culminating in more aggressive disease. For example, the immature and leaky vasculature of tumor-associated blood vessels is dependent on TGFβ expression, secretion or activity of MMPs. A local angiogenic cytokine and protease network directly or indirectly effects endothelial cell growth, motility and survival by release of endothelial cells from the membrane, thus propelling the dissolution of mature vessels in the vicinity of the tumor[121,123,202,203].

TGFβ is known to inhibit the growth of many haemotopoietic cell lines, just as it does in the epithelial cells of tissue[23,204]. In normal mesenchymal fibroblasts or in keratinocytes (skin cells), supra-physiological TGFβ blocks progression through G1 resulting in a senescence-like arrest or cytostastis[121]. Not surprisingly, these cells, next to epithelial cells in various stages of transformation, are used for studying escape of proliferation. The study of how cells read TGFβ signals has allowed the delineation of transcriptional programs that mediate specific TGFβ effects. Though mutations in canonical TGFβ components are common in cancer, we still have only a rudimentary understanding of the downstream effectors involved in proliferation arrest. It is undeniably imperative to identify them, as this may provide us a much-desired clearer view on the process coupling tumor initiation to tumor progression. Escape of a TGFβ-dependent proliferation-arrest is therefore an intensely studied area in cancer research.

Genetic screens

Instead of educated guessing, an unbiased approach toward identifying new players in specific pathways is by using genetic screening for a distinct cellular phenotype. RNAi- or loss of function screens are very popular these days, but an alternative and approach is cDNA retroviral screening for gain of phenotype escape of a p53-dependent proliferation-arrest.

Retroviral cDNA library screen for bypass of senescence

We live in an era that sees a flood of sequence data emerging[205,206]. Various genome projects have exposed a wealth of information, but this is accompanied by the lack of knowledge of how the newly discovered genes may be functioning. Establishing efficient DNA sequencing of normal and tumor tissue is relatively easy, but the challenge lies in assigning functions[207]. Cell culture gives a reductionists' view of cells in a 2D arrangement, and provides us a defined experimental setup in which to investigate the phenotypic consequence of genetic alterations. Through environmental or added stress a wide spectrum of cell fates as differentiation or apoptosis can be elicited, which then can be used as readout for the activity of specific genes involved. The power of this technique in understanding rudimentary cellular or tumor-suppressive responses is well documented[208-210].

Mammalian cells comprise a variety of different cell populations, both genetically and phenotypically. They can be derived from dissimilar tissue and their growth and differentiation status can therefore vary widely from cell line to cell line. Nevertheless, of many proliferation-arrest related phenotypes we by now have established some of the main players, and their significance in tumorigenesis is evident[56].

Escape of the cell cycle arrest will lead to growth, which can easily be scored in a colony formation, or low density, analysis. The more

robust the system, the less off-target effects or leakiness will interfere with scoring true inhibitors of p53 signaling. An important trick is to get the cells expressing foreign or multiple copies of a genome-wide spectrum of tissue- or (patho)physiology-specific genes (cDNA 'libraries'), which may lead to an interaction with the p53-dependent observed phenotype. There are various cDNA libraries from either normal or pathological tissue available, enlarging the possibilities of asking tissue- or disease-specific questions. These cDNA collections are produced via a reverse transcriptase reaction of the transcribed RNA of particular cells, and are thus a copy of their gene-activity. After transduction of the target cells with a cDNA library and plating them out, it can be as easy as just waiting whether some p53-interacting gene may indeed show up by enforcing uncontrolled growth. Subsequent isolation of the crucially involved and transduced DNA sequences may uncover previously unappreciated yet highly influential players in p53 signaling, and perhaps shed light on the versatility of p53-interacting pathways.

Outline of this thesis

It is becoming increasingly clear that cancer is not only caused by the cell autonomous mutations that deregulate proliferation and survival of the cancer cell itself, but also by a dynamic interplay with the surrounding tissue (stroma). Decades after the first proposals that disease outcome may be dependent on a close collaboration between tumor cells and their local environment we now acknowledge the causality of such cross-talk in tumor progression. In this thesis, I describe studies on genes that play a role in escape from proliferation-arrest. These findings provide new insights into the interplay between tumor and stroma.

Chapter 1 is a general introduction on some of the cell cycle- and microenvironment-associated oncogenes and tumor-suppressors of our interest, and how our ideas were shaped in studying proliferation arrest.

Chapter 2 describes that PAI-1 is a critical target in execution of p53-dependent cellular senescence in fibroblasts and gives details on how this may be established mechanistically. We suggest that PAI-1 is an extra-cellular gatekeeper of p53-dependent proliferation, by regulating growth factor dependent nuclear retention of Cyclin D1.

Chapter 3 explains that PAI-1 is also a critical target for that other tumor-suppressor, TGFβ, in induction of a G1 arrest. It further confirms findings described in Chapter 2 that enhanced PI3K-PKB mitogenic signaling can render a cell insensitive to multiple tumor-suppressive pathways. We suggest that the cytostatic activity of TGFβ is dependent on PAI-1.

Chapter 4 shows that enhanced LPA receptor signaling instructs Rho GTPases to induce bypass of a p53-dependent arrest in a genetic screen in mouse cells. We suggest that genes involved in cell-morphological traits may directly be connected to cell cycle inhibition via E2F regulation.

Chapter 5 is a discussion of our findings, especially in the light of novel observations that loss of PAI-1 function induces not only a cell autonomous but also a cell non-autonomous immortalizing effect. Furthermore, we speculate on the meaning of our findings that suggest newly identified direct connections between cell cycle regulators and micro-environmental cues.

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There are many reasons which send men to the Poles, and the Intellectual Force uses them all. But the desire for knowledge for its own sake is the one which really counts and there is no field for the collection of knowledge which at the present time can be compared to the Antarctic. Exploration is the physical expression of the Intellectual Passion. And I tell you, if you have the desire for knowledge and the power to give it physical expression, go out and explore. If you are a brave man, you will do nothing: if you are fearful you may do much, for none but cowards have need to prove their bravery. Some will tell you that you are mad, and nearly all will say 'What is the use?'. For we are a nation of shopkeepers, and no shopkeeper will look at research which does not promise him a financial return within a year. And so you will sledge nearly alone, but those with whom you sledge will not be shopkeepers: that is worth a good deal. If you march your Winter Journeys, you will have your reward, so long as all you want is a penguin's egg.

Apsley Cherry-Garrard, *The Worst Journey in the World – an account of the Antarctic expedition by Sir Robert Falcon Scott 1910-1913* (1923)

CHAPTER 2

Plasminogen Activator Inhibitor-1 is a Critical Downstream Target of p53 in the Induction of Replicative Senescence

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Plasminogen Activator Inhibitor-1 is a Critical Downstream Target of p53 in the Induction of Replicative Senescence

Roderik M. Kortlever*, Paul J. Higgins# and René Bernards*

p53 limits the proliferation of primary diploid fibroblasts by inducing a state of growth arrest named replicative senescence, a process which protects against oncogenic transformation and requires integrity of the p53 tumor suppressor pathway[1-3]. However, little is known concerning the downstream target genes of p53 in this growth-limiting response. We report here that suppression of the p53 target gene encoding plasminogen activator inhibitor-1 (*PAI-1*) by RNA interference leads to escape from replicative senescence both in primary mouse embryo fibroblasts and primary human BJ fibroblasts. PAI-1 knockdown results in sustained activation of the PI3K-PKB-GSK3β pathway and nuclear retention of cyclin D1, consistent with a role for PAI-1 in regulating growth factor signaling. In line with this, we find that the PI3K-PKB-GSK3β-cyclin D1 pathway is also causally involved in cellular senescence. Conversely, ectopic expression of PAI-1 in proliferating p53-deficient murine or human fibroblasts induces a phenotype displaying all the hallmarks of replicative senescence. Our data indicate that PAI-1 is not merely a marker of senescence, but both necessary and sufficient for the induction of replicative senescence downstream of p53.

Introduction

Primary murine fibroblasts activate the $p19^{ARF}$-p53 tumor suppressor pathway during prolonged culturing *in vitro*, which induces a post mitotic state referred to as replicative senescence. Senescence can be overcome by loss of either $p19^{ARF}$, p53, or the combined loss of all three retinoblastoma family proteins (Ref. [1,2]). Proliferation of fibroblasts is induced by growth factors which activate cyclin-dependent kinases (CDKs), and in turn inactivate pRb's growth limiting ability[2], a G1 cell cycle checkpoint, which is often deregulated in cancer[3]. It is not clear which of the many downstream p53 target genes is responsible for the p53-dependent induction of replicative senescence. An attractive candidate is the CDK inhibitor $p21^{CIP1}$. However, mouse

* Division of Molecular Carcinogenesis and Center for Biomedical Genetics The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.
# Center for Cell Biology & Cancer Research, Albany Medical College, MC-165, 47 New Scotland Avenue, Albany, NY 12208, USA.

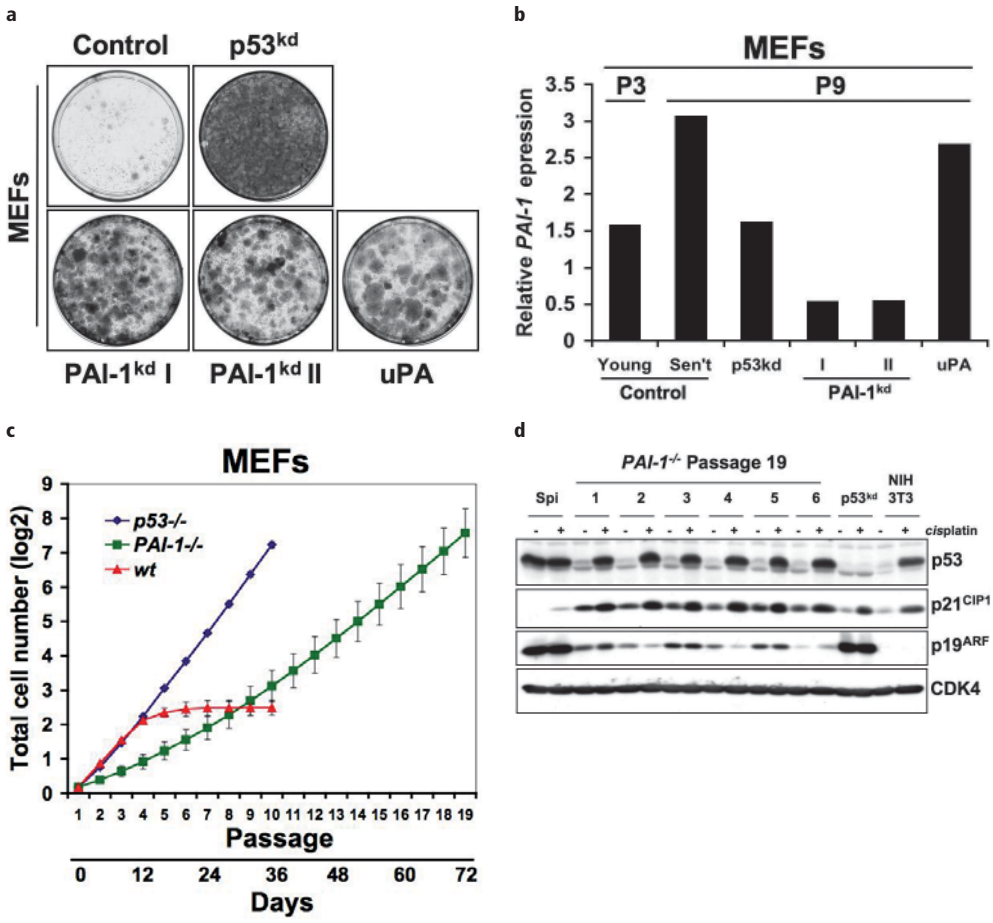

Figure 1 PAI-1 loss induces senescence-bypass in primary mouse fibroblasts.

(a) Colony formation assay in primary MEFs over-expressing indicated constructs. A knockdown vector for p53 was used as a positive control.

(b) Relative *PAI-1* expression analyzed by quantitative real-time PCR on indicated post-senescent polyclonal cell lines. P3 is young passage 3, and P9 is senescent (sen't) passage 9 MEFs.

(c) Growth curves of *PAI-1*$^{-/-}$, *p53*$^{-/-}$ and wild-type (wt) MEFs. Per genotype the mean (+/- SD) of 6 independent cultures is shown.

(d) Western blot analysis of a spontaneously immortal (spi), six independent *PAI-1*$^{-/-}$ passage 19, p53^{kd} and p16^{INK4A}/p19^{ARF}-deficient immortal NIH3T3 cell lines showing status of p53 and its targets p19^{ARF} and p21^{CIP1} after *cis*platin-induced DNA damage. CDK4 is loading control. Uncropped full scans are shown in Supplementary Information.

embryo fibroblasts (MEFs) knockout for *p21*^{CIP1} are not immortal[4].

In this study we identify an unexpected causal role for the uPA/PAI-1 system in the induction of replicative senescence. The serpin and extra-cellular matrix (ECM)-associated protein PAI-1 is a direct target of p53[5,6], is up-regulated in aging fibroblasts *in vivo* and *in vitro*, and considered a marker of replicative senescence[7-9]. PAI-1 inhibits the activity of the secreted protease urokinase type plasminogen activator (uPA) by forming a stable complex. uPA expression can cause cells to progress through G1 into S phase[10], most likely by activating a mitogenic signaling cascade by increasing bioavailability of growth factors.

Results

To study the role of PAI-1 in replicative senescence, we generated two independent retroviral vectors that target murine PAI-1 for suppression through RNA interference[11]. When primary MEFs were infected with either PAI-1 knockdown (kd) construct (PAI-1^{kd} I, PAI-1^{kd} II), we observed senescence bypass in a colony formation assay (Figure 1a). Since inhibition of PAI-1 expression leads to activation of uPA[12,13], we asked whether over-expression of uPA also caused immortalisation. Retrovirus-mediated over-expression of pro-uPA was as efficient as PAI-1 knockdown in causing immortalisation of MEFs (Figure 1a). To assess *PAI-1* levels in infected MEFs, we serially passaged p53^{kd}, PAI-1^{kd}, or uPA over-expressing cells until they had become post-senescent at passage 9 (P9) (*i.e.* when control MEFs were senescent) and observed a significant reduction in *PAI-1* mRNA expression in PAI-1^{kd} MEFs by quantitative real-time PCR (QRT-PCR) (Figure 1b). Since PAI-1 is a transcriptional p53 target[5,6] and p53 is activated during replicative senescence, PAI-1 is highly expressed in senescent MEFs[9]. Accordingly, P9 MEFs expressed more *PAI-1* than passage 3 (P3) or p53^{kd} cells, which showed comparable *PAI-1* mRNA levels (Figure 1b). uPA activity is downstream of PAI-1 and p53, and consequently immortal cells over-expressing uPA harbour *PAI-1* expression levels similar to those seen in P9 MEFs (Figure 1b). When tested in a long-term cell proliferation assay, PAI-1^{kd} also extended the proliferative capacity of MEFs far beyond that of wild-type cells (see Supplementary Information, Figure S1a). Spontaneous immortalisation of MEFs can either be caused by mutation of p53 or by loss of p19^{ARF} expression[14,15,16], which was not observed in PAI-1^{kd} cells as judged by their normal p53-dependent DNA damage response (see Supplementary Information, Figure S1b). Since p19^{ARF} levels in PAI-1^{kd} cells are comparable to those seen in wild-type senescent cells, we conclude that p19^{ARF} expression is not lost after knockdown of *PAI-1*. Consistent with these observations, *PAI-1* knockout (*PAI-1*$^{-/-}$) MEFs proliferated well beyond the senescence checkpoint, albeit at a slower rate than *p53*$^{-/-}$ MEFs (Figure 1c). Importantly, six independent immortal *PAI-1*$^{-/-}$ MEF cell lines showed normal p53 function after DNA damage exposure (Figure 1d).

Next we sought to gain insight into the molecular pathway(s) involved in the immortalisation of MEFs after PAI-1 knockdown or uPA over-expression. The tumor-suppressors p16^{INK4A} and p21^{CIP1} were induced in P9 MEFs and immortal PAI-1^{kd} or uPA over-expressing cells, indicating that the senescence stress pathway is activated in these MEFs (Figure 2a). Interestingly, we observed a sharp increase in cyclin D1 levels in both non-proliferating P9 and proliferating PAI-1^{kd} or uPA over-expressing MEFs, which may neutralize the high levels of p21^{CIP1} (Figure 2a). We asked whether signaling through PI3K, PKB (also known as AKT) and GSK3β was involved. Activation of

PI3K and subsequent full activation of PKB by phosphorylation on Serine 473 leads to a de-activating phosphorylation of GSK3β on Serine 9 by PKB[17,18]. GSKβ controls cyclin D1 localization and degradation through an inhibitory phosphorylation on Threonine 286 and loss of this inhibitory phosphorylation protects cyclin D1 from nuclear exclusion and degradation[19]. Therefore, mitogenic signaling through PI3K-PKB-GSK3β influences cyclin D1 stability and its nuclear activity, leading to cell-cycle progression[2]. uPA induces growth factor-related PI3K-PKB signaling[20] and, as a result, uPA activity might result in nuclear retention of cyclin D1 by inducing loss of GSK3β activity through phosphorylation on Serine 9. When we determined cyclin D1 localization in aging MEFs, we noticed a striking but gradual nuclear exclusion of cyclin D1 (Figure 2b) correlating with the decline in growth rate (note that wild-type MEFs became fully senescent at passage 8 (see Supplementary Information, Figure S2a), as confirmed by staining for senescence-associated acidic β-galactosidase[21] (SA-β-Gal., data

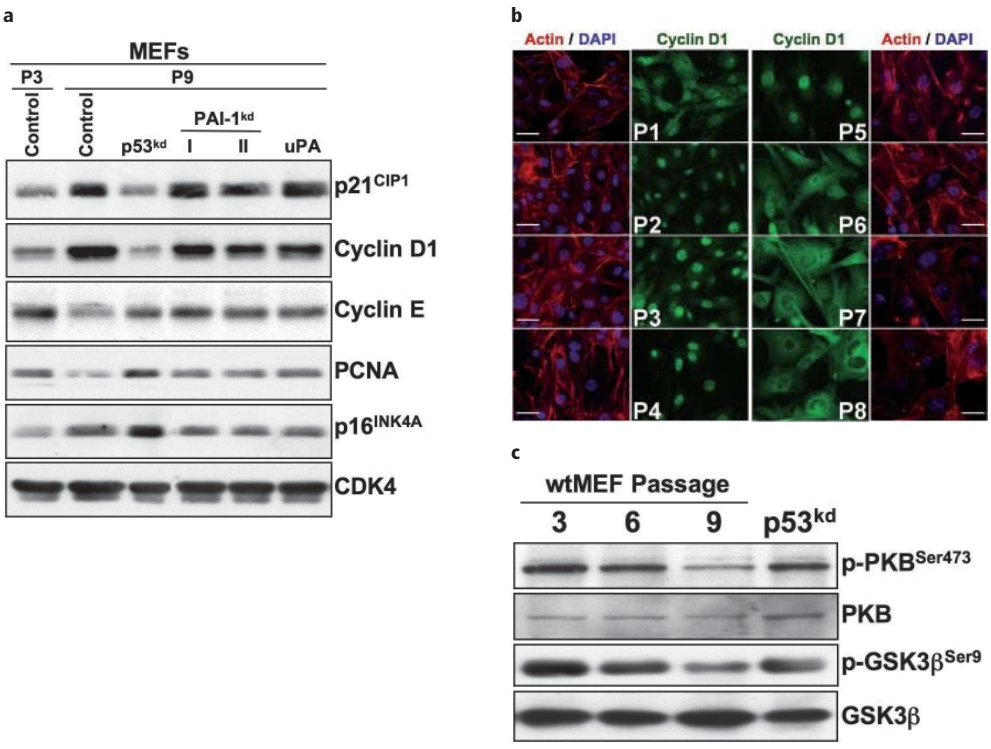

Figure 2 MEFs reduce PKB activation and exclude cyclin D1 from the nucleus during replicative senescence.

(a) Western blot analysis of P3, P9, $p53^{kd}$, PAI-1^{kd}, or uPA over-expressing MEFs for cell cycle related proteins. PCNA and CDK4 are proliferation and loading controls, respectively.

(b) Qualitative immunofluorescence analysis of serially passaged MEFs, passage 1 (P1) to passage 8 (P8), for cyclin D1 expression. Bar represents 50 μm.

(c) Expression of phosphorylated PKB or GSK3β related to unphosphorylated fraction of the same proteins in passage 3, 6, and 9 MEFs and post-senescent $p53^{kd}$ cells as analyzed by western blot.

not shown)). Furthermore, during serial passaging, wild-type MEFs show a progressive decline in activation of PKB by loss of phosphorylation and a gradual increase of activation of GSK3β by loss of inhibitory phosphorylation (Figure 2c). In contrast, immortal PAI-1^{kd} or uPA over-expressing MEFs had sustained PKB-GSK3β signaling (see Supplementary Information, Figure S2b). We therefore conclude that during replicative senescence cyclin D1 is excluded from the nucleus and stabilized in the cytosol, correlating with decreased growth-rate and down-regulation of PKB-GSK3β signaling. The high levels of cyclin D1 we found in senescent cells (Figure 2a) are unexpected because GSK3β activity induces not only nuclear exclusion but also the turn-over of cyclin D1[19]. Significantly, we also observed high levels of cyclin D1 in senescent human BJ fibroblasts (Figure 5c). Apparently, the turnover of cyclin D1 is different in senescent cells as compared to cycling NIH3T3 cells[19].

We next asked whether immortalisation of MEFs by PAI-1 knockdown might be the result of activated PI3K-PKB-GSK3β signaling. We retrovirally transduced primary MEFs with constitutively active mutants of PI3K (p110α^{CAAX}: ca-PI3K) or PKB (myr-PKB: ca-PKB), a retroviral knockdown shRNA construct for GSK3β, or retroviral expression constructs for wild-type cyclin D1 (D1) or non-degradable cyclin D1 (T286A-cyclin D1: TA-D1), and determined their immortalizing potential in a colony formation assay. The TA-D1 mutant has become refractory to phosphorylation by GSK3β and is therefore constitutively nuclear[19]. We found that ca-PI3K, ca-PKB, GSK3β^{kd}, or TA-D1 (but not wild type D1) are individually capable of immortalizing wild-type MEFs (Figure 3a). Furthermore, we found no loss of $p19^{ARF}$ expression or evidence for mutation of p53 in any of the immortalised MEFs (data not shown). When tested in a long-term proliferation assay, over-expression of a ca-PI3K, ca-PKB, GSK3β^{kd}, or TA-D1 (but not wild type D1) construct also induced a senescence-bypass in MEFs (Figure 3b). Again, immortalisation was not accompanied by loss of $p19^{ARF}$ or p53 function (data not shown). As previously seen in PAI-1^{kd} or uPA over-expressing MEFs (see Figure 2a), we noticed induction of $p21^{CIP1}$ protein levels in the post-senescent polyclonal cell lines expressing a ca-PI3K, ca-PKB, GSK3β^{kd}, or TA-D1 construct (see Supplementary Information, Figure S2c). Furthermore, we noticed high PKB-GSK3β signaling in ca-PI3K or ca-PKB over-expressing cells, and reduced GSK3β expression in GSK3β^{kd} cells (see Supplementary Information, Figure S2d). Our results suggest that enforced constitutive activation of PI3K-PKB signaling, reduction of GSK3β activity, or nuclear retention of cyclin D1 is sufficient to bypass senescence in MEFs downstream of p53. Accordingly, immunofluorescence analysis of post-senescent polyclonal cell lines of MEFs immortalised with the various constructs used in this study revealed nuclear localization of endogenous cyclin D1 when compared to senescent wild-type or HA-tagged cyclin D1 expressing MEFs (Figure 3c, and see Supplementary Information, Figure S2e).

To test whether reduction of PI3K-PKB-GSK3β signaling is sufficient for the induction of senescence, we over-expressed antagonists of this mitogenic signaling route in p53-depleted cells. We generated retroviral cDNA expression constructs for human PAI-1 (which is not targeted by the mouse PAI-1^{kd} vectors), mouse PTEN and GSK3β. PTEN is a potent tumor suppressor often deleted in cancer that blocks the activation of PI3K[22]and exerts its effect in part by regulating nuclear availability of cyclin D1[23]. When over-expressed in $p53^{kd}$ or PAI-1^{kd} cells, PAI-1, PTEN, and GSK3β were all individually able to induce senescence as evidenced by a flat-cell morphology and positive stain-

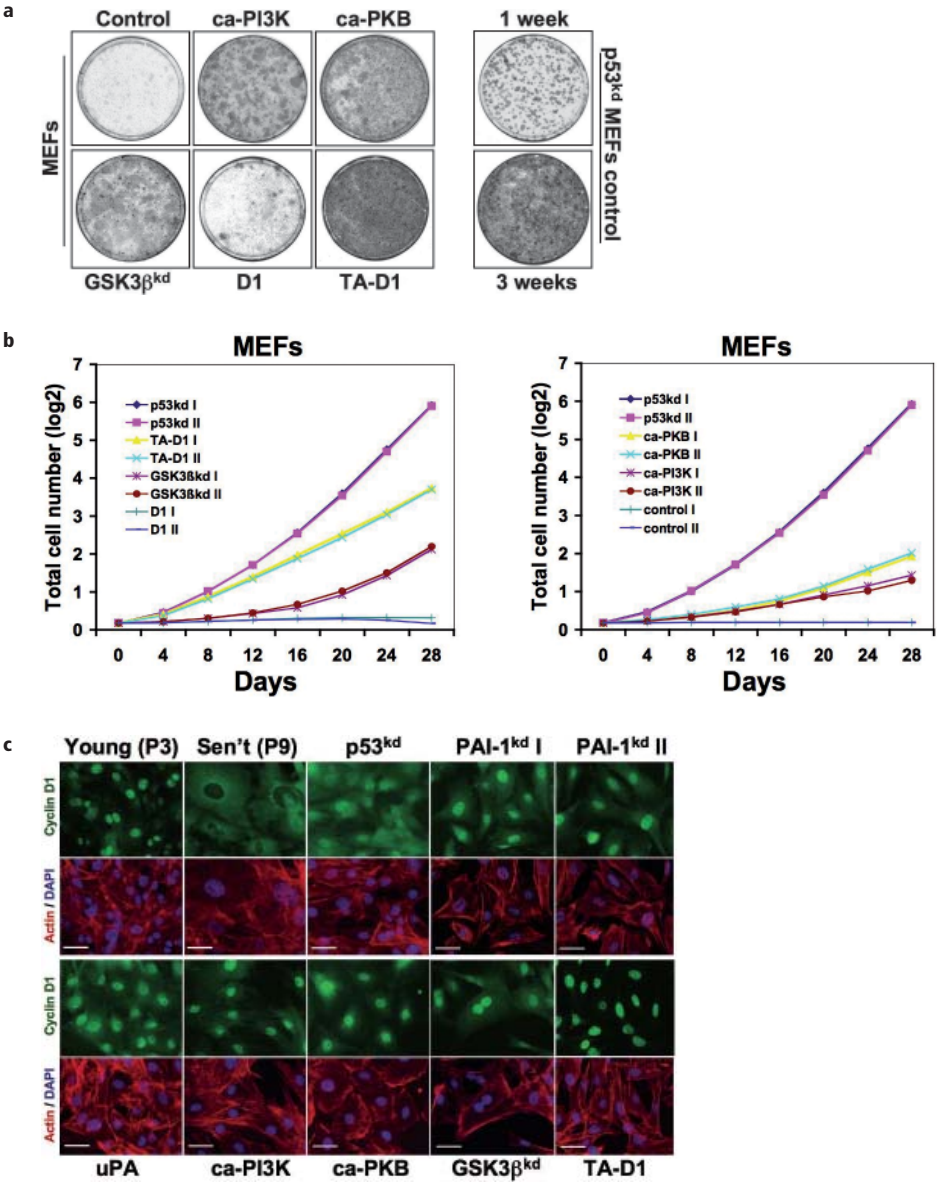

Figure 3 Sustained PI3K-PKB signaling or nuclear retention of cyclin D1 induces senescence bypass.

(a) Colony formation assay of primary MEFs infected with indicated retroviral constructs. Immortalizing efficiency controls are $p53^{kd}$ MEFs stained after 1 or 3 weeks.

(b) Growth curves of various depicted immortalizing constructs versus wild-type cyclin D1 (D1) infected MEFs. Over-expression of $p53^{kd}$ or control vector are positive and negative controls, respectively. Shown are results of two independent infections per construct (I, II).

(c) Qualitative immunofluorescence analysis for cyclin D1 of post-senescent MEFs immortalised with indicated constructs, and control P3 and P9 MEFs. Bar represents 50 μm.

ing for SA-β-Gal. (Figure 4a, b, c, d). To test whether cyclin D1 is an essential target downstream of PAI-1, we chose to over-express PAI-1 in $p53^{-/-}$ MEFs, MEFs over-expressing TA-D1, or pocket protein deficient triple knockout cells ($pRb^{-/-}/p107^{-/-}/p130^{-/-}$: TKO). The immortal TKO controls lack retinoblastoma family function and have therefore sustained E2F activity[24,25]. In normal fibroblasts pRb (family)-E2F-mediated repression is required for cell cycle exit in response to $p19^{ARF}$-p53 activation[26]. Over-expression of TA-D1 in MEFs might therefore resemble the phenotype seen in TKO cells by blocking retinoblastoma family function. Fig 4e shows that PAI-1 induced an arrest in $p53^{-/-}$ cells, but not when these cells also expressed TA-D1 or were pocket protein deficient. These results suggests that in the presence of a wild-type cyclin D1 protein PAI-1 is able to induce an arrest, but not when cyclin D1 is constitutively nuclear and insensitive to GSK3β.

The senescence response of human fibroblasts is in first instance (M1 checkpoint) primarily dependent on p53 and, like in MEFs, PAI-1 is up-regulated during aging and a marker of senescence in these cells[7,27]. When primary human BJ fibroblasts were infected with either one of two independent human-specific PAI-1 knockdown constructs ($PAI\text{-}1^{kd}$ I, $PAI\text{-}1^{kd}$ II) we observed an M1 senescence-bypass as judged in a long-term growth assay (Figure 5a). As in MEFs, over-expression of uPA also induced a bypass of senescence in primary BJ cells, albeit with lower efficiency (Figure 5a). When the post-senescent and proliferating population doubling (PD) 68 $PAI\text{-}1^{kd}$ were assayed for *PAI-1* levels, we noticed reduction to levels even below those observed in young PD 30 BJ fibroblasts (Figure 5b). *PAI-1* levels in PD 68 uPA over-expressing BJ cells were similar to those seen in senescent cells, in agreement with the notion that uPA acts downstream of p53. $PAI\text{-}1^{kd}$ or uPA over-expressing fibroblasts have notably higher levels of $p21^{CIP1}$ than $p53^{kd}$ cells (Figure 5b, c), consistent with the notion that $PAI\text{-}1^{kd}$ mediates senescence-bypass downstream of p53. That p53 is wild type in the $PAI\text{-}1^{kd}$ or uPA over-expressing BJ fibroblast is also supported by their normal response to DNA damage (data not shown). As observed in MEFs, we found induction of cyclin D1 in $PAI\text{-}1^{kd}$ and uPA over-expressing post-senescent BJ cells, as well as in non-proliferating senescent BJ cells (Figure 5c). Furthermore, we noticed an increase in cytoplasmic cyclin D1 in aging BJ fibroblasts, which we found to be associated with $p21^{CIP1}$ (see Supplementary Information, Figure S4a, b, c), though the nuclear to cytoplasmic transition of cyclin D1 seems to be not as pronounced as in aging MEFs. Importantly, over-expression of murine PAI-1, PTEN, or GSK3β in immortal human $p53^{kd}$ or $PAI\text{-}1^{kd}$ cells induced an arrest and SA-β-Gal. staining (Figure 5d, e), indicating that PAI-1 expression and down-regulation of the PI3K-PKB-GSK3β signaling route are also sufficient for induction of senescence in human fibroblasts downstream of p53 and PAI-1. Taken together, we conclude that PAI-1 is necessary and sufficient for senescence in human BJ fibroblasts. Since $p21^{CIP1}$ is an essential p53 target in the senescence response of human fibroblasts[28,29], we asked if simultaneous knockdown of both $p21^{CIP1}$ and PAI-1 would induce a more efficient senescence-bypass than either one alone. We found that knockdown of the expression of either *PAI-1* or $p21^{CIP1}$ results in a less efficient senescence-bypass than seen with knockdown of p53 (see Supplementary Information, Figure 5a, b). Interestingly, simultaneous knockdown of PAI-1 and $p21^{CIP1}$ resulted in a more efficient bypass of the arrest than knockdown of p53 itself (see Supplementary Information, Figure S5a, b, c). We conclude that PAI-1 and $p21^{CIP1}$ are both relevant downstream targets of p53 in the induction of senescence in human

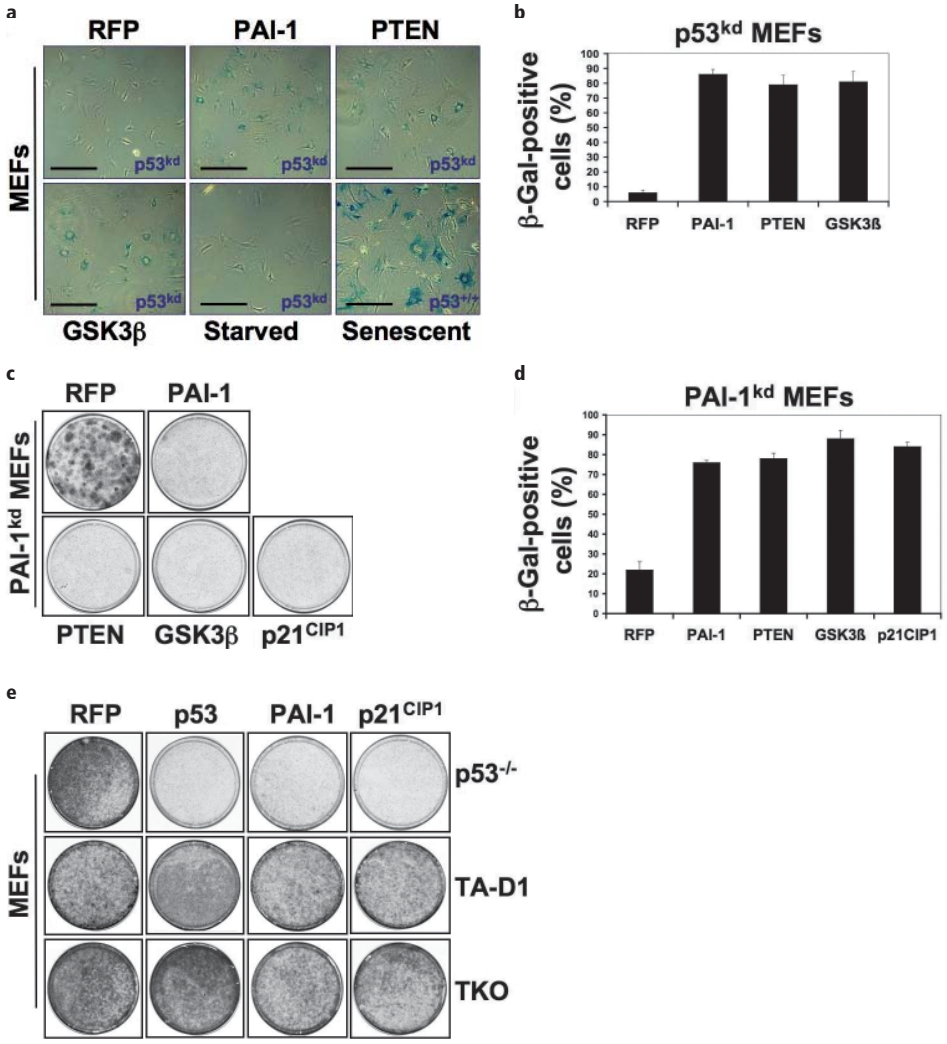

Figure 4 PAI-1 expression is sufficient for the induction of replicative senescence.

(a) PAI-1, PTEN, or GSK3β over-expression induces senescence in $p53^{kd}$ MEFs as indicated by staining for senescence-associated β-galactosidase (SA-β-Gal.). Control cells are mock-infected, serum depleted (starved) and wild-type senescent MEFs. Bar represents 400 μm.

(b) Quantification of SA-β-Gal. positive $p53^{kd}$ cells after retroviral over-expression of constructs as indicated in (**a**). Shown is the mean (+/- SD) per plate/infection.

(c) Colony formation assay in immortal PAI-1^{kd} MEFs after retroviral over-expression of indicated constructs. $p21^{CIP1}$ and red fluorescent protein (RFP) are positive and negative controls, respectively.

(d) Quantification of SA-β-Gal. positive PAI-1^{kd} MEFs after retroviral over-expression of PAI-1, PTEN, GSK3β, $p21^{CIP1}$, or an RFP control. Shown is the mean (+/- SD) per plate/infection.

(e) Colony formation assay in immortal $p53^{-/-}$, TA-D1, or TKO (retinoblastoma family triple knockout; $pRb^{-/-}/p107^{-/-}/p130^{-/-}$) fibroblasts infected with indicated retroviral over-expression constructs.

fibroblasts, as evidenced by the effects of their combined knockdown.

Discussion

Here we show that PAI-1 is a critical downstream target of p53 in the senescence response of both aging mouse and human diploid fibroblasts. Our data indicate that p53 controls growth factor-dependent proliferation by upregulating PAI-1, leading to down-regulation of PI3K-PKB signaling and nuclear exclusion of cyclin D1. Conversely, we find that loss of *PAI-1* expression or uPA over-expression in MEFs confers resistance to the anti-proliferative activity of p53 by inducing sustained PI3K-PKB signaling and cyclin D1 nuclear retention. Our data are consistent with a model in which PAI-1 acts to limit cyclin/CDK activity during the induction of replicative senescence and suggest a role for PAI-1 as a secreted gatekeeper of fibroblast proliferative capacity (Figure 5f).

We find that the mitogen-stimulated PI3K-PKB route is causally involved in the senescence-bypass of fibroblasts, and that over-expression of their antagonist PTEN[22], can reverse this process. It has been reported that the levels of PTEN are crucial in determining the senescence response of fibroblasts: Partial loss of PTEN confers a proliferative advantage, whereas acute loss of all PTEN induces senescence[30]. Consistent with this, we find that somatic knockdown of PTEN in wt MEFs results in senescence bypass (data not shown) and constitutive activation of the PI3K-PKB signaling route, albeit not to the degree seen in ca-PI3K or ca-PKB over-expressing cells (see Supplementary Information, Figure S3b). In apparent conflict with our data, which show senescence bypass by active PKB, it has been reported that over-expression of an active PKB resulted in induction of senescence. However, when these cells were followed over 6 days, proliferation was not entirely lost in the infected population[30]. We have selected PKB-infected wild type MEFs over a longer period of time, and consequently enriched for proliferating cells with potentially only moderately enhanced PKB activity. Together, these data support the notion that slightly elevated levels of PKB or partial loss of PTEN results in enhanced proliferation whereas highly elevated PKB or complete loss of PTEN induces senescence. This is reminiscent of what has been observed in RAS signaling, where over-expression of an activated *RAS* oncogene induces senescence, whereas activated RAS expressed at physiological levels confers a growth advantage[31].

PAI-1 is induced by a variety of growth factors and is a target of *c-Myc*[7,32]. uPA transcription and its extra-cellular activity are regulated by growth factor signaling and proteases. Induction of PAI-1 may therefore be part of a growth factor-stimulated negative feedback loop that becomes constitutively activated by p53 in aging fibroblasts. As a consequence, senescent fibroblasts may induce a state of growth factor unresponsiveness by secreting PAI-1. It is therefore possible that induction of *PAI-1* by p53 or disturbance of the uPA/PAI-1 levels influences intra-tumoral heterotypic signaling and local tumor microenvironment. This is particularly noteworthy since uPA and PAI-1 are causally involved in wound healing, angiogenesis and metastasis[12,33], processes dominantly regulated by cell-cell signaling[34]. Furthermore, uPA is secreted by stromal fibroblasts and myofibroblasts at the invasive front in breast and prostate cancer[35], and loss of uPA can result in reduced metastasis in mouse models[36,37]. Since it is becoming increasingly clear that stromal tissue is an indispensable player in neoplastic transformation and metastasis[38,39], our observations may lead to a better understanding of the role of fibroblasts in cancer.

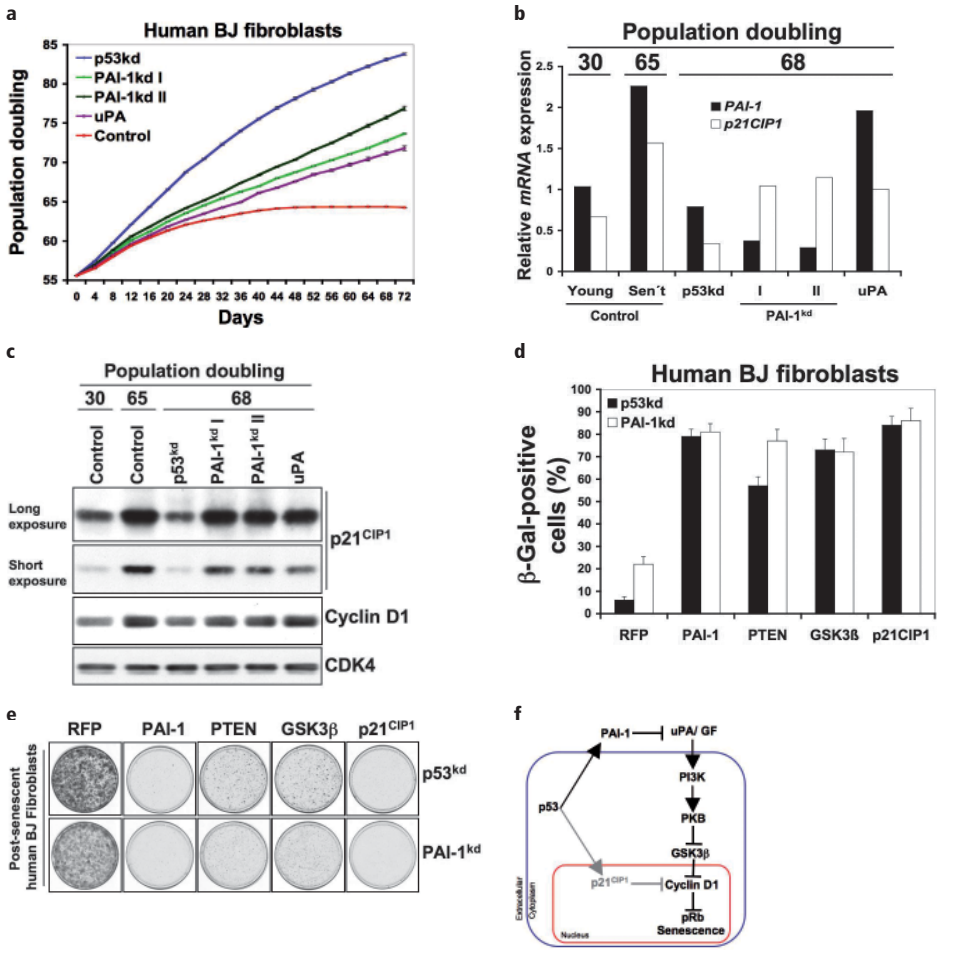

Figure 5 PAI-1 is necessary and sufficient for senescence in human BJ fibroblasts.

(a) Growth curves of primary human BJ fibroblasts over-expressing the indicated constructs. Per genotype the mean (+/- SD) of 3 independent cultures is shown.

(b) Relative *PAI-1* and *p21^{CIP1}* expression analyzed by quantitative real time PCR on indicated post-senescent BJ cell lines and control-infected young or senescent (sen't) BJ fibroblasts.

(c) Western blot analysis of cells from (**a**) for p53-target p21^{CIP1}, and cell cycle related protein cyclin D1. CDK4 is loading control.

(d) Quantification of SA-β-Gal. positive post-senescent PAI-1^{kd} or p53^{kd} BJ fibroblasts after retroviral over-expression of PAI-1, PTEN, GSK3β, p21^{CIP1}, or an RFP control. Shown is the mean (+/- SD) per plate/infection.

(e) Colony formation assay in post-senescent p53^{kd} or PAI-1^{kd} BJ fibroblasts infected with indicated retroviral over-expression constructs. RFP is a negative control.

(f) Proposed model of senescence in fibroblasts. p53 induces PAI-1 and p21^{CIP1} during aging in culture. PAI-1 antagonizes uPA/GF (growth factor) signaling to cyclin D1 via PI3K-PKB-GSK3β and p21^{CIP1} blocks cyclin D1 activity directly. The PAI-1-cyclin D1 connection is dominant over p21^{CIP1} activity and controls induction of the senescence response downstream of p53 and upstream of pRb.

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Author contributions. RK and RB conceived and designed the experiments. RK performed the experiments. PH contributed materials. RK and RB analyzed the data. RK and RB wrote the paper.

Competing interests statement: The authors declare that they have no competing financial interests.

Materials and Methods

Antibodies and Vectors For western blotting, antibodies against $p16^{INK4A}$ (M156), $p21^{CIP1}$ (F5, C19), SP1 (PEP2), cyclin D1 (H295, M20), cyclin E (M20), PCNA (PC-10), PKB/Akt1 (C20), p53 (DO-1), HA (Y11), and CDK4 (C22) were from Santa Cruz Biotechnology (Santa Cruz, CA), anti p-PKB (Ser473; #9271), anti p-GSK3β (Ser9; #9336) and HSP90 (#4874) from Cell Signaling (Beverly, MA), anti $p19^{ARF}$ (Ab 80-100) from Abcam, anti-GSK3β (610201) from BD Pharmingen (San Jose, CA), anti Phosphotyrosine (PY20) from Calbiochem and anti p53 (Ab7) from Oncogene Research Products (Boston, MA). Flag-tagged mouse cDNAs for PAI-1, GSK3β, PTEN, or human PAI-1 were generated by PCR amplification and cloned into pLZRS-IRES-zeocin. Mouse cDNA for uPA was generated by PCR amplification of pro-uPA and cloned into pBABEpuro. The production of siRNAs in MEFs, BJ or tsLT-hTERT-BJ fibroblasts was achieved using the pRETRO-SUPER vector[11]. For the generation of mouse PAI-1 knockdown constructs the following 19-mer sequences were used: PAI-1 I, 5'-GAACAAGAATGAGATCAGT-3'; PAI-1 II, 5'-GTTGGGCATGCCTGACATG-3'. For the generation of human PAI-1 knockdown constructs the following 19-mer sequences were used: PAI-1 I, 5'-CTGACTTCACGAGTCTTTC-3'; PAI-1 II, 5'-CCTGGGAATGACCGACATG-3'. The 19-mer sequences used for knockdown of mouse p53 or GSK3β have been described elsewhere[40,41], just as the 19-mer sequences used for knockdown of human p53 or $p21^{CIP1}$ (Ref [29]). Control infections were performed with non-functional hairpin or red fluorescent protein (RFP) vectors.

Cell culture, transfection and retroviral infection Mouse embryo fibroblasts (MEFs), primary and tsLT hTERT human BJ fibroblasts and Phoenix cells were cultured in DMEM (Gibco) supplemented with 8% heat-inactivated fetal bovine serum (Perbo),2mM L-Glutamine and penicillin/streptomycin (Gibco). Transfections were performed with the calcium-phosphate precipitation technique. Retroviral supernatants were produced by transfection of Phoenix packaging cells. Viral supernatants were filtered through a 45 μm Millex® HA filter (Millipore, Carrigtwohill, Co. Cork, Ireland), and infections were performed in the presence

of 4 μg ml^{-1} polybrene (Sigma). Drug selections in MEFs or BJs were performed with 1 μg ml^{-1} puromycin, 50 μg ml^{-1} hygromycin, or 100 μg ml^{-1} zeocin.

Colony formation assays Wild-type MEFs were infected with shRNA or cDNA constructs at passage 3, selected, and at passage 5 50.000 cells were seeded onto 10 cm plates and stained after 3 weeks. 50.000 $p53^{kd}$ control MEFs were seeded onto 10 cm plates and stained after 1, 2 or 3 weeks. Immortal PAI-1^{kd} MEFs were infected, after 72 hours plated under low density (50.000 cells in a 10 cm plate), and 2 weeks later stained. *$p53^{-/-}$*, TA-D1 or TKO MEFs were infected, 48 hours after infection 50.000 cells were seeded onto 10 cm plates, and stained after 1 week. Human post-senescent $p53^{kd}$ or PAI-1^{kd} BJ fibroblasts were infected with cDNA constructs, plated under low density (100.000 cells in a 10 cm plate), and stained after 2 weeks. Human tsLT BJ fibroblasts were infected at 32°C, after 48 hours 100.000 cells were seeded per 10 cm plate and shifted to 39°C, and stained after 2 weeks. For all colony formations representative examples of at least three independent experiments are shown.

Growth curves MEFs were infected with retroviral shRNA or cDNA expression constructs at passage 3, selected, and at passage 5 150.000 cells were plated in a 6 cm dish (time = 0 days). Every 4 days cells were counted, and 150.000 cells were replated. A MEF passage as we define it in this paper represents 4 days in culture. 150.000 *PAI-$1^{-/-}$*, *$p53^{-/-}$*, or wild-type MEFs were plated in a 6 cm dish at passage 1, and every 4 days cells were counted and 150.000 cells were replated. Human primary BJ fibroblasts at population doubling 53 were infected, selected, and 150.000 cells were plated in a 6 cm dish (time = 0 days). Every 4 days cells were counted and 150.000 cells were replated. Human tsLT BJ fibroblasts were infected, shifted to 39°C after 48 hours, and 150.000 cells were plated in a 6 cm dish (time = 0 days). Every 6 days cells were counted and 150.000 cells were replated. p53 status of control, senescent or post-senescent primary MEFs, BJ fibroblasts or tsLT BJ fibroblasts was checked by DNA-damage induced p53 activation by overnight addition of 0,5 mM *cis*-platin and western blotting for p53 and its targets $p19^{ARF}$ and $p21^{CIP1}$ in MEFs, or $p21^{CIP1}$ and Bax in BJ fibroblasts. Total cell amounts in all growth curves were displayed as cumulative over time. For all growth-curves representative examples of at least two independent experiments are shown.

Quantitative real time PCR From immortal post-senescent MEF cell lines plus passage 3 and 9 control MEFs, control or immortalized human BJ fibroblasts and of immortalised tsLT BJ fibroblasts at 39°C or controls at 32°C or 39°C total RNA was isolated with TRI-Zol© (Invitrogen, Carlsbad, CA) according to manufacturer instructions. QRT-PCR was performed on an ABI Prism 7700 with Assays-on-Demand™ (Applied Biosystems) for mouse *PAI-1* and *TBP* as a control housekeeping gene, or for human *PAI-1* or *$p21^{CIP1}$* with *GAPDH* as a control housekeeping gene. QRT-PCR results in tsLT BJ fibroblasts are the mean (+/- SD) of three independent cell lines per genotype. For all QRT-PCRs representative examples of at least two independent experiments are shown.

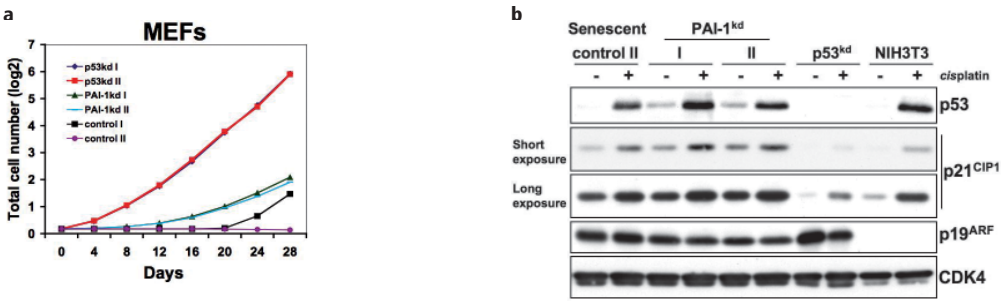
Immunofluorescence microscopy Cells were plated on 8-well chamber slides (Nutacon; Leimuiden, The Netherlands) and cultured overnight after which they were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 15 minutes, permeabilized with 0,2% Triton-X (Sigma), blocked, and incubated with anti-cyclin D1 (M20) from Santa Cruz Biotechnology (Santa Cruz, CA). Actin was stained

with rhodamine-conjugated Phalloidin (Invitrogen) and the nucleus with DAPI (Roche). Images were obtained using a Modified Zeiss Axiovert 100M (SP LSM 5) cooled CCD fluorescence microscope with a Plan-APOCHROMAT, 1.0 NA, 40x oil immersion objective plus a Zeiss single excitation-triple emission filter set 40 with a KP650 red blocking filter on a Photometrics MAC 200A camera and SmartCapture V2.0 software. For all immunostainings representative examples of at least three independent experiments are shown.

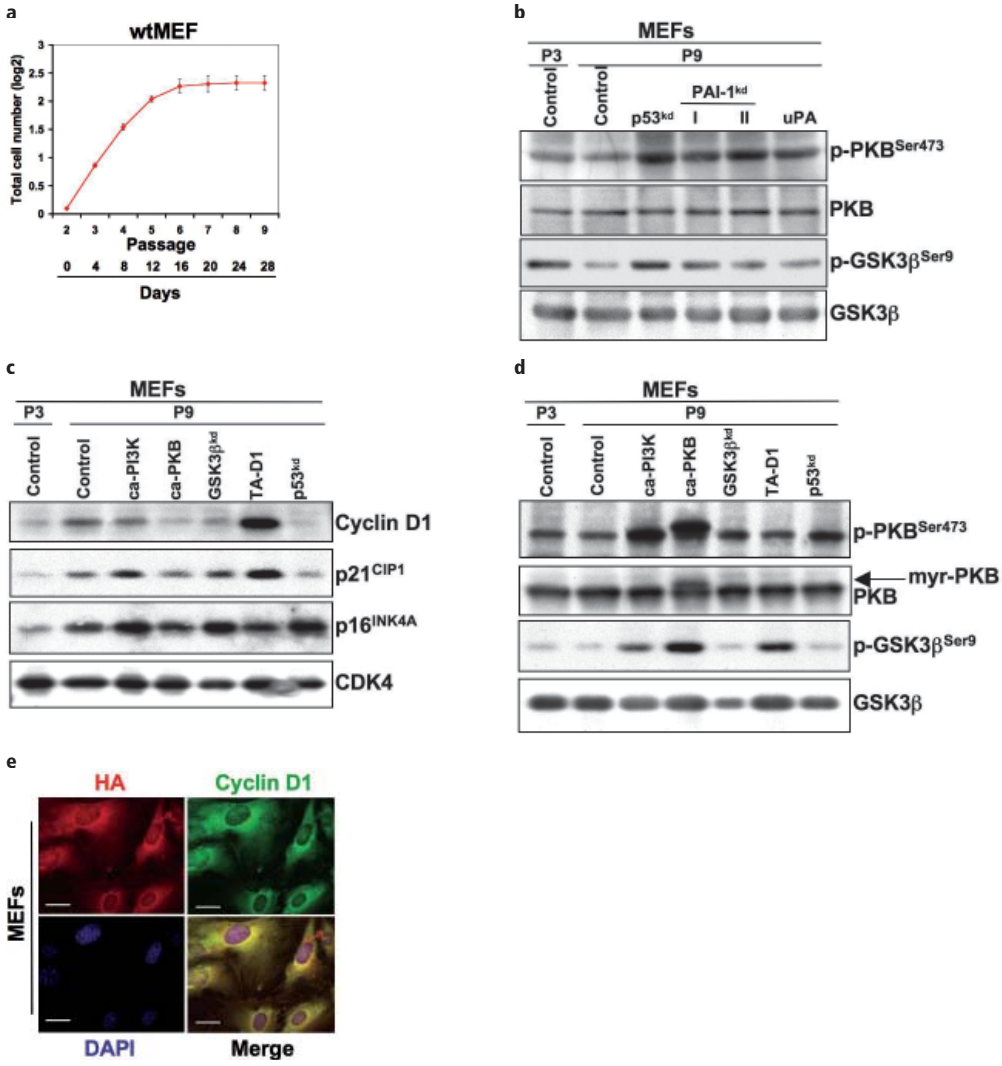
Acidic β-galactosidase staining $p53^{kd}$ or PAI-1^{kd} MEFs were infected, after 4 days plated under low density (50.000 cells in a 10 cm plate), and 24 hours later stained overnight for senescence-associated acidic β-galactosidase according to Dimri *et al* [21]. Human post-senescent immortal $p53^{kd}$ or PAI-1^{kd} BJ fibroblasts were infected, after 5 days plated under low density (100.000 cells in a 10 cm plate), and 48 hours later stained overnight for acidic β-galactosidase according to Dimri *et al* [21]. Per plate three independent groups of 300 cells were counted for SA-β-Gal staining. For all SA-β-Gal. stainings representative examples of at least two independent experiments are shown. Images were obtained using a Zeiss Axiovert 25 microscope with A-Plan 10x or LD A-plan 20x objectives on a Canon Powershot G3 14x zoom camera.

Western blotting Selected cells were lysed in RIPA buffer (50 mM Tris pH 8; 150 mM NaCl; 1% NP40; 0,5% DOC; 0,1% SDS). 20, 40 or 80 micrograms of protein was separated on 8-12% SDS-polyacrylamide gel by electrophoresis and transferred to polyvinylidine difluoride membranes (Millipore). Western blots were probed with the indicated antibodies. For all western blots representative examples of at least two independent experiments are shown.

Co-immunoprecipitations Total cell lysates were isolated with ELB buffer (0.25 M NaCl, 0.1% NP40, 50 mM Hepes pH 7.3) supplemented with Complete protease inhibitors (Roche). Cytoplasmic fractions of BJ cells were isolated with Nuclear and Cytoplasmic Extraction kit NE-PER® (Pierce Biotechnology Inc., Rockford, IL) according to manufacturer instructions. Lysates were incubated with protein-A sepharose beads (Amersham, Pharmacia Biotech) coated with anti-Cyclin D1 (M20, Santa Cruz Biotechnology, Santa Cruz, CA). Analysis of Cyclin D1 or Cyclin D1-associated proteins was done by western blotting the precipitates from cytoplasmic and total lysates.



Supplementary Figure S1 PAI-1^{kd} induces senescence-bypass in primary MEFs retaining p19^{ARF}-p53 signaling.
(a) Long-term proliferation curves of p53^{kd}, PAI-1^{kd}, or non-functional shRNA control infected MEFs. Shown are the results of two independent experiments per construct (I, II).
(b) Western blot analysis for p53 and its targets p19^{ARF} and p21^{CIP1} using cell lines depicted in (**a**) after *cis*platin-induced DNA-damage. NIH3T3 cells are immortal p16^{INK4A}/p19^{ARF}-deficient controls.



Supplementary Figure S2 Retention or induction of PKB-GSK3β signaling in immortal MEFs.

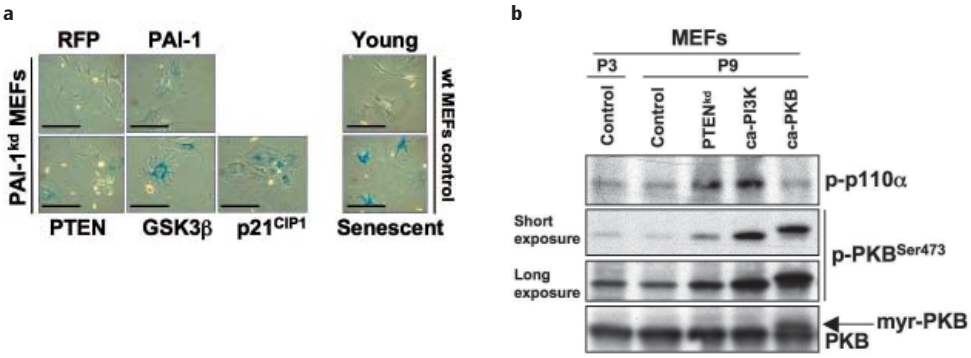
(a) Proliferation curves of 6 independently isolated cultures of wild-type MEFs. Mean value (+/- SD) is indicated.

(b) Expression of phosphorylated PKB or GSK3β related to unphosphorylated fraction of the same proteins in P3, P9, $p53^{kd}$, PAI-1^{kd}, or uPA over-expressing cells as analyzed by western blot.

(c) Western blots of protein samples of indicated cell lines with antibodies for tumor suppressors $p16^{INK4A}$ or $p21^{CIP1}$, or cyclin D1. CDK4 is a loading control.

(d) Western blots for the same set of cell lines as in (**c**) for phospho-specific PKB and GSK3β, as compared to unphosphorylated fractions of the same proteins.

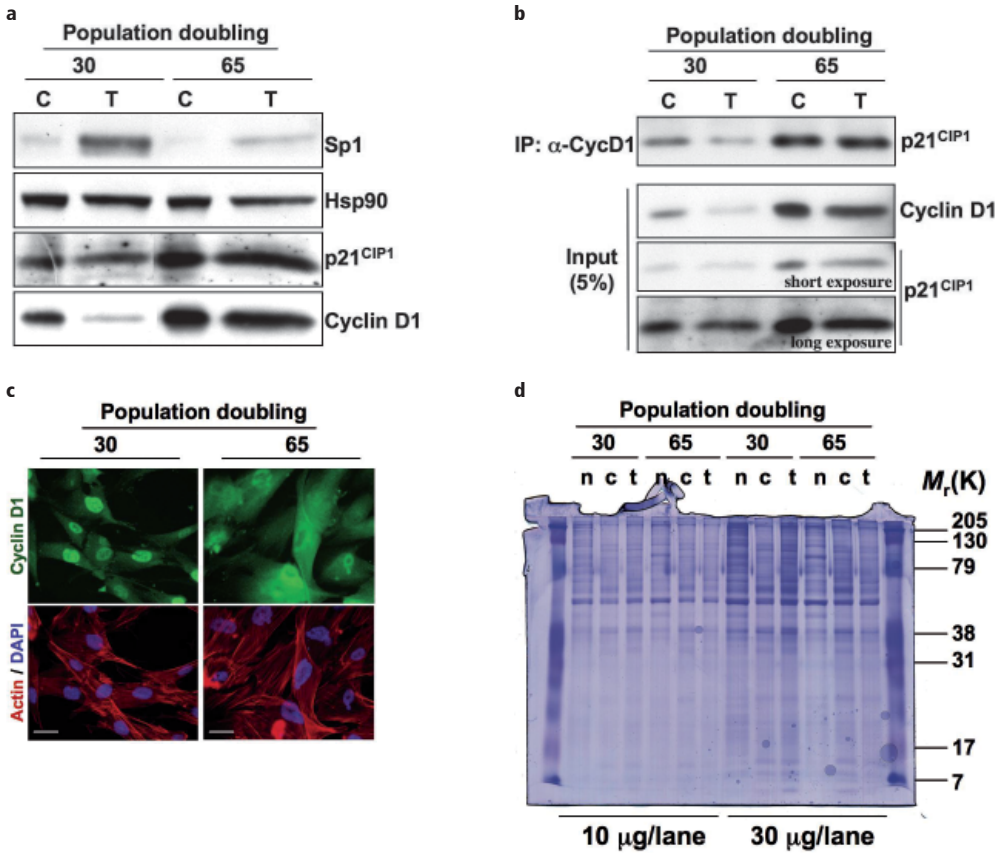
(e) Qualitative immunofluorescence analysis for HA-tag (HA) and cyclin D1 in senescent MEFs over-expressing HA-tagged cyclin D1. Bar represents 50 μm.



Supplementary Figure S3 PAI-1 is sufficient for induction of senescence in MEFs

(a) PAI-1, PTEN, GSK3β, or $p21^{CIP1}$ over-expression induces senescence in $PAI\text{-}1^{kd}$ MEFs as indicated by staining for senescence-associated β-galactosidase (SA-β-Gal). Staining controls are young and senescent wild-type MEFs. Scale bar represents 250 μm.

(b) Expression of phosphorylated p110α (catalytic subunit of PI3K) and PKB related to unphosphorylated fraction of the same protein in P3, P9, $PTEN^{kd}$, ca-PI3K or ca-PKB over-expressing cells as analyzed by western blot.



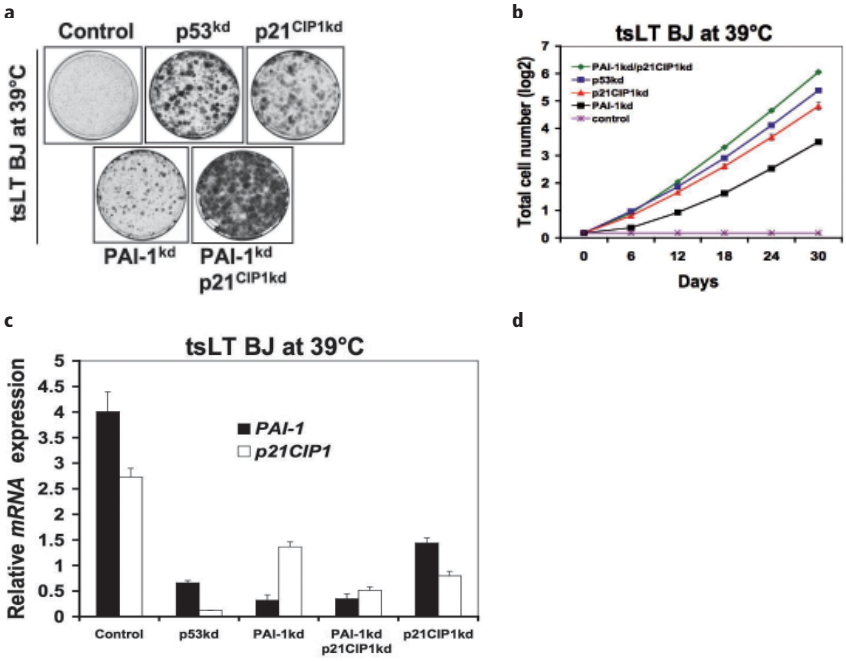
Supplementary Figure S4 Cytoplasmic co-localization of cyclin D1 and p21^{CIP1} in aging primary BJ fibroblasts.

(a) Western blot of cytoplasmic (C) and total (T) protein lystates from primary young passage doubling 30 and senescent passage doubling 65 BJ fibroblasts probed for indicated proteins. Sp1 is a nuclear protein and Hsp90 a cytoplasmic fraction control.

(b) Immunoprecipitation with cyclin D1 antibody in protein lysates from (a) immunoblotted for p21^{CIP1}.

(c) Qualitative immunofluorescence analysis of serially passaged BJs, passage doubling 30 or passage doubling 65, for cyclin D1 expression. Bar represents 50 μm.

(d) Coomassie staining of SDS gel with 10 or 30 μg of nuclear (n), cytoplasmic (c) or total (t) protein from young population doubling (PD) 30 or senescent PD 65 primary BJ fibroblasts, for equal loading.



Supplementary Figure S5 PAI-1 and p21^{CIP1} collaborate in senescence response downstream of p53.

(a) Colony formation assay of depicted constructs in conditionally immortalized tsLT hTERT BJ fibroblasts[29]. Control is a non-functional shRNA. These cells enter into a p53-dependent proliferation arrest when shifted to the non-permissive temperature (39°C). Importantly, these cells show virtually exact characteristics as primary BJ fibroblasts when challenged through assays as described in Figure 5a-e (data not shown).

(b) Long term growth curves at the non-permissive temperature of 39°C of cells over-expressing depicted constructs. Per genotype the mean (+/- SD) of 3 independent cultures is shown. *PAI-1* and *p21^{CIP1}* are both p53 target genes and knockdown of the expression of either gene alone results in a less efficient senescence bypass than seen with knockdown of p53 alone (see also (a)).

(c) Quantitative real-time PCR analysis of relative expression of *PAI-1* and *p21^{CIP1}* in cell lines depicted in (**b**). As in primary BJ fibroblastst (see Figure5b), we noticed reduction of *PAI-1* mRNA in p21^{CIP1kd} cells and reduction of *p21^{CIP1}* mRNA in PAI-1^{kd} cells, suggesting that loss of either gene influences transcription of the other.

The shock of the real. For a little while we are again able to see, as the child does, a world of marbles. For a few moments we discover that nothing can be taken for granted, for if this ring of stone is marvelous then all which shaped it is marvelous, and our journey here on earth, able to see and touch and hear in the midst of tangible and mysterious things-in-themselves, is the most strange and daring of all adventures.

…

As for the "solitary confinement of the mind", my theory is that solipsism, like other absurdities of the professional philosopher, is a product of too much time wasted in library stacks between the covers of a book, in smoke-filled coffeehouses (bad for the brains) and conversation-clogged seminars. To refute the solipsist or the metaphysical idealist all that you have to do is take him out and throw a rock at his head: if he ducks he's a liar. His logic may be airtight but his argument, far from revealing the delusions of living experience, only exposes the limitations of logic.

…

If a man's imagination were not so weak, so easily tired, if his capacity for wonder not so limited, he would abandon forever such fantasies of the supernal. He would learn to perceive in water, leaves and silence more than sufficient of the absolute and marvelous, more than enough to console him for the loss of ancient dreams.

Edward Abbey, *Desert Solitaire – a season in the wilderness.* (1968)

CHAPTER 3

TGFβ Requires its Target Plasminogen Activator Inhibitor-1 for Cytostatic Activity

Submitted for publication

TGFβ Requires its Target Plasminogen Activator Inhibitor-1 for Cytostatic Activity

Roderik M. Kortlever and René Bernards

The cytokine TGFβ has strong antiproliferative activity in most normal cells, but contributes to tumor progression in the later stages of oncogenesis. It is not fully understood which TGFβ target genes are causally involved in mediating its cytostatic activity. We report here that suppression of the TGFβ target gene encoding plasminogen activator inhibitor-1 (*PAI-1*) by RNA interference leads to escape from the cytostatic activity of TGFβ both in human keratinocytes (HaCaTs) and primary mouse embryo fibroblasts (MEFs). Consistent with this, *PAI-1* knockout MEFs are also resistant to TGFβ growth arrest. Conversely, we show that ectopic expression of PAI-1 in proliferating HaCaT cells induces a growth arrest. *PAI-1* knockdown does not interfere with canonical TGFβ signaling as judged by SMAD phosphorylation and induction of *bona fide* TGFβ target genes. Instead, knockdown of *PAI-1* results in sustained activation of PKB. Significantly, we find that constitutive PKB activity leads to evasion of the growth-inhibitory action of TGFβ. Our data are consistent with a model in which induction of *PAI-1* by TGFβ is critical for the induction of proliferation arrest.

Introduction

Transforming growth factor-β (TGFβ) controls many processes, including cell proliferation, differentiation and stress responses, as well as the production of extra-cellular matrix (ECM)-associated proteins and secretory growth factors[1,2]. TGFβ is a ubiquitous and potent growth-inhibitory cytokine in various cell types as, for example, keratinocytes and other epithelial cells and acts through binding to a transmembrane receptor, which in turn phosphorylates SMAD2 and SMAD3 proteins. These activated SMADs then translocate to the nucleus with binding partner SMAD4 and this complex regulates a wide range of target genes, among which both pro- and anti-proliferative genes[3,4]. The cytostatic effect is mediated by the induction of cyclin dependent kinase (CDK) inhibitors $p21^{CIP1}$ and $p15^{INK4b}$, and is dependent on retinoblastoma (pRb) function[1,5-7]. This way growth factor-dependent CDK activity is inhibited and

Division of Molecular Carcinogenesis, Center for Cancer Genomics and Center for Biomedical Genetics, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands.

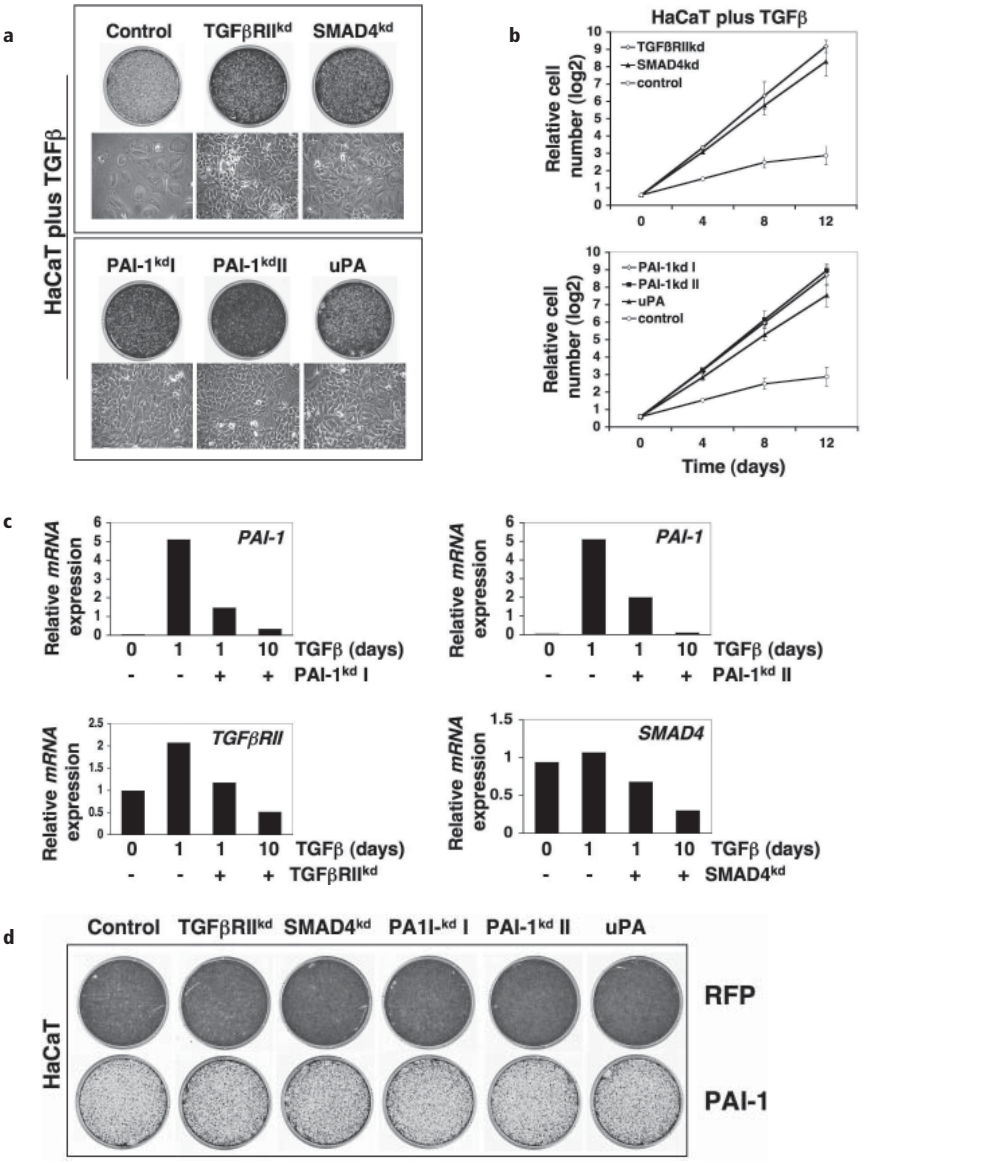


Figure 1 PAI-1 is required for TGFβ-induced cytostasis in human keratinocytes. *A.* Colony formation assay in HaCaT cells over-expressing indicated constructs treated with 200 pM TGFβ for 7 days. TGFβRII and SMAD4 knockdown vectors are positive controls. A non-functional shRNA is used as a negative control. Also shown are phase contrast images of the cells after 7 days in culture in the presence of TGFβ. *B.* Growth curves of HaCaT cells expressing indicated constructs in the presence of 200 pM TGFβ. A non-functional shRNA is used as a negative control. *C. PAI-1*, *TGFβRII* and *SMAD4* mRNA levels as quantified by QRT-PCR in PAI-1, TGFβRII and SMAD4 shRNA-expressing HaCaT cells. Shown are relative levels after 1 or 10 days of TGFβ treatment compared to no treatment and a non-functional shRNA control. *D.* Colony formation assay in TGFβ-bypassing HaCaT cells expressing a non-functional shRNA or depicted constructs and infected with a Red Fluorescent Protein (RFP) expressing control vector or retroviral PAI-1 cDNA.

progression through the cell cycle prevented. In addition, TGFβ induces genes implicated in invasion and cell motility, which includes secreted ECM-associated proteins as plasminogen activator inhibitor-1 (*PAI-1*) and multiple matrix metalloproteases (MMPs)[3,8,9].

The pleiotropic actions of TGFβ have important consequences in cancer. Abortion of the cytostatic response may provide cancer cells with a selective advantage including invasive behavior[10]. Noticeably, resistance to the growth-inhibitory activity of TGFβ is often found in cancer, for example by mutations that inactivate the TGFβ receptors or SMAD signal transducers[3,8,11]. In addition, selective mutation of genes required for cytostatic TGFβ responses may also occur in the presence of apparently normal TGFβ receptor and SMAD function. Identifying these factors is essential to better understand how during tumor progression cells evade the growth-limiting response to TGFβ.

One of the target genes of SMAD activity that is potently induced by TGFβ in keratinocytes, fibroblasts, epithelial, and endothelial cells is *PAI-1* ([3,12]). Similar to TGFβ, PAI-1 is involved in extra-cellular matrix homeostasis and angiogenesis, and associated with cancer progression[13,14]. Recently we found that *PAI-1* is a critical target of tumor-suppressor p53 in the induction of cellular senescence in fibroblasts[15]. We therefore asked whether *PAI-1* might also be causally involved in the cytostatic activity of TGFβ. Our present results reveal an unexpected role for *PAI-1* in mediating the anti-proliferative effects of TGFβ.

Results

Cytostatic TGFβ activity in human keratinocytes requires PAI-1

Human HaCaT cells are arrested in the G1 phase of the cell cycle in response to TGFβ, while exhibiting strongly induced *PAI-1* levels[3,5,12]. We asked whether the induction of *PAI-1* might be causally involved in the TGFβ-induced proliferation arrest. TGFβ receptor type II (TGFβRII) and SMAD4 knockdown vectors were used as positive controls[3]. We stably infected HaCaT cells with retroviral constructs expressing two independent PAI-1 shRNAs (to reduce the chance of scoring for an off-target effect), a non-functional shRNA (used as a negative control), or the TGFβRII or SMAD4 shRNAs. We also transduced HaCaT cells with a vector encoding a cDNA for urokinase type plasminogen activator (uPA) as this secreted protease and growth factor acts downstream of its antagonist PAI-1. Consequently, enhanced uPA expression may override the *PAI-1* induction by TGFβ. Figure 1*A* shows that colony formation of parental HaCaT cells exposed to 200 pM TGFβ was severely inhibited, but that knockdown of *PAI-1* by two independent shRNA vectors or expression of uPA resulted in escape from growth arrest which was at least as potent as that caused by knockdown of TGFβRII or SMAD4 (Figure 1*A*, Supplemental Figure S1*A*).

A similar result was seen when growth curves were made of HaCaT cells having these knockdown vectors (Figure 1*B*). There was no significant growth difference between the various cell lines in the absence of TGFβ (data not shown). To assess the knockdown efficiency in TGFβ-resistant cells we determined the mRNA levels of *PAI-1*, *TGFβRII*, and *SMAD4* in cells expressing the respective knockdown constructs by quantitative real-time PCR (QRT-PCR). Following stimulation with 200 pM TGFβ for one day, *PAI-1* mRNA levels were highly induced in normal HaCaT cells, which was greatly suppressed in PAI-1^{kd} cells. This became more exacerbated when cells were grown in TGFβ medium for ten days (Figure 1*C*). There was reduction of *TGFβRII* and *SMAD4* in TGFβRII^{kd} and SMAD4^{kd} cells, respectively, and progressive

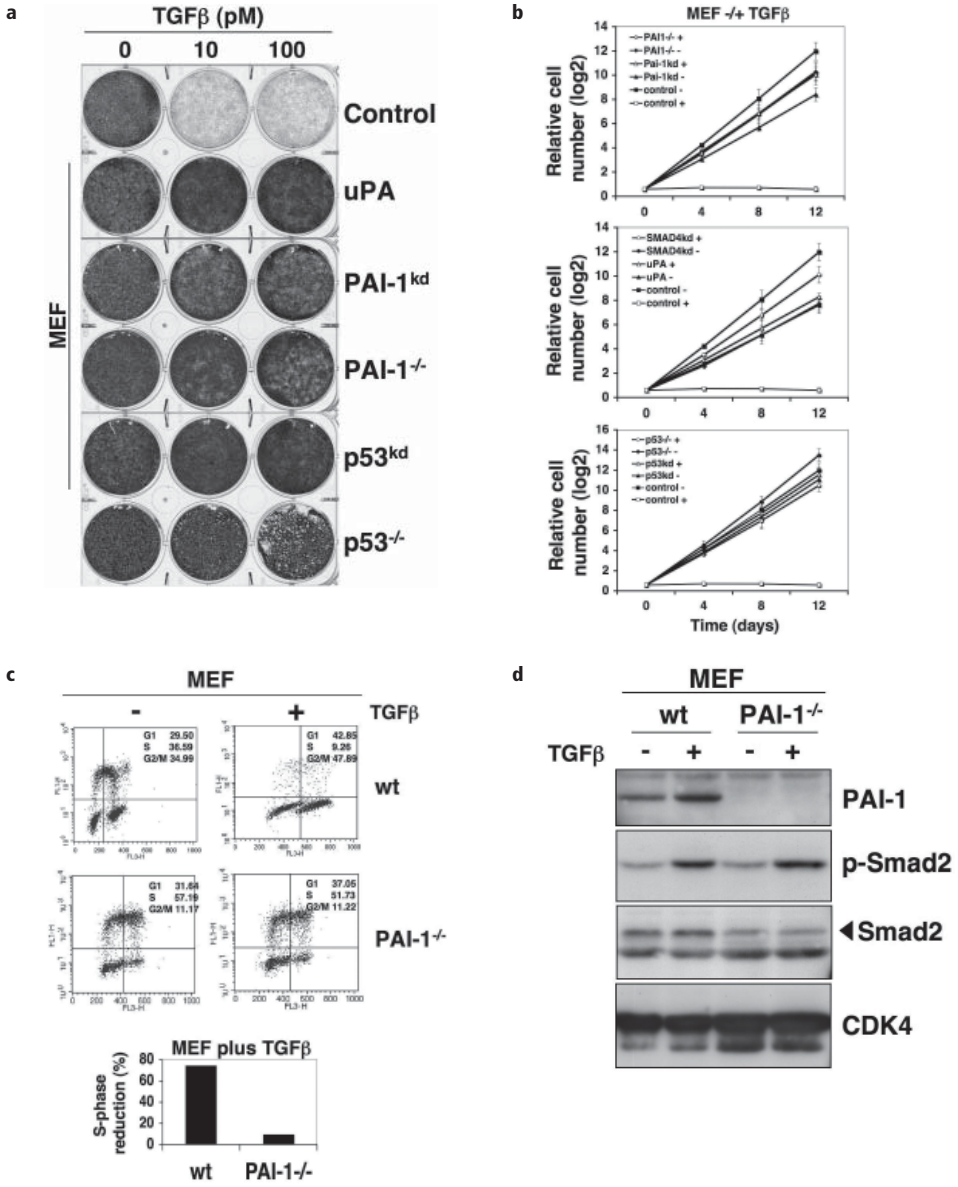


Figure 2 PAI-1 loss induces bypass of the cytostatic response to TGFβ in mouse embryo fibroblasts. *A.* Colony formation assay in MEFs over-expressing uPA, indicated *PAI-1* knockdown constructs, or knockout for *PAI-1* or *p53*, in the absence and presence of TGFβ for 7 days. A non-functional shRNA is used as a negative control. *B.* Growth curves of MEFs expressing indicated knockdown constructs or knockout for *PAI-1* or *p53*, in the absence and presence of TGFβ. SMAD4 and non-functional shRNAs are used as a positive and a negative control, respectively. *C.* Cell cycle analysis of wild-type (wt) and PAI-1$^{-/-}$ MEFs treated overnight without (-) and with (+) TGFβ and subsequently incubated with PI/BrdU. FACS analysis shows cell cycle profile and strong reduction in BrdU incorporation in wild-type but not PAI-1$^{-/-}$ MEFs. *D.* Western blot analysis for PAI-1 and SMAD2 activation of young proliferating wild-type (wt) or PAI-1$^{-/-}$ MEFs treated overnight without (-) and with (+) TGFβ. Probed was for PAI-1, phospho-SMAD2 compared to normal SMAD2 levels, and for loading control CDK4.

reduction over time when grown in the presence of TGFβ (Figure 1*C*).

Next we asked whether over-expression of a mouse cDNA for PAI-1, which is insensitive to knockdown by the human PAI-1 shRNAs[15], could mimic TGFβ activity and induce a cell cycle arrest. We analyzed in a colony formation assay whether the growth of TGFβ-resistant cells expressing the various knockdown constructs was influenced by ectopic expression of murine PAI-1, as compared to infection with a red fluorescent protein (RFP) control. In all cases the cells were growth arrested (Figure 1*D*), and the inhibition of proliferation by PAI-1 expression was approximately comparable between the various cell lines (Supplemental Figure S1*B*). We conclude that loss of *PAI-1* expression results in bypass of the cytostatic activity of TGFβ, and that ectopic *PAI-1* expression can be sufficient for induction of a growth arrest in human keratinocytes.

Cytostatic TGFβ activity in mouse fibroblasts requires PAI-1

MEFs grown under low density are also sensitive to the cytostatic effect of TGFβ, which is pRb dependent[6]. Therefore we analyzed the consequences of both *PAI-1* knockdown and knockout in MEFs on the anti-proliferative effect of TGFβ. Young rapidly growing primary MEFs were used as a TGFβ-sensitive control, while MEFs ablated for *p53* (both knockdown and knockout) served as positive controls for TGFβ growth arrest bypass[16]. The wild-type MEFs or MEFs knockdown or knockout for *PAI-1*, or over-expressing uPA exhibited normal p53 function (data not shown). When analyzing their proliferation in a colony formation assay in the presence of 10 or 100 pM TGFβ, we found that over-expression of uPA and knockdown or knockout of *PAI-1* induced a bypass of a growth arrest, which was comparable to the knockdown or knockout of *p53* (Figure 2*A*). That *PAI-1*$^{-/-}$ MEFS are unresponsive to TGFβ strongly supports the notion that the TGFβ-bypass by *PAI-1* knockdown is not an off target effect. Furthermore, when followed over time in a growth curve assay in the presence of 100 pM TGFβ, we found that over-expression of uPA or knockdown or knockout of *PAI-1* or *p53* in MEFs also resulted in bypass of growth-inhibition (Figure 2*B*). SMAD4^{kd} (also with normal p53 function) was added as a positive control[17] (Figure 2*A*, Supplemental Figure S2). We conclude that PAI-1 is also required for TGFβ-induced cytostasis in mouse embryo fibroblasts.

To study whether PAI-1 affects the short-tem effects of TGFb on cell cycle, we subjected both wild type MEFs and *PAI-1*$^{-/-}$ MEFs to TGFb overnight and analyzed the fraction of S-phase cells by measuring BrdU incorporation. Figure 2*C* shows that wild type MEFs had an immediate and strong descrease in S phase cells in response to TGFb, whereas the *PAI-1*$^{-/-}$ MEFs were virtually non-responsive to TGFb growth inhibition. The absence of a growth inhibitory response in the *PAI-1*$^{-/-}$ MEFs was not due to a lack of TGFb signaling, as SMAD2 activation by phosphorylation was unaffected by PAI-1 loss (Figure 2*D*). We conclude that in MEFs, PAI-1 is critical for both short-term and long-term anti-proliferative responses to TGFb.

PAI-1 knockdown does not interfere with canonical TGFβ signaling

We next investigated to what extent reduction of *PAI-1* levels in HaCaTs and MEFs would influence canonical TGFβ signaling, which is dependent on receptor-mediated SMAD2 activation by phosphorylation and specific CDK-inhibitor target gene activation[4]. After stimulation with 200 pM TGFβ for ten days, HaCaT cells having knockdown of PAI-1 or SMAD4 or over-expression of uPA showed unaltered SMAD2 phosphorylation when compared to the control (Figure 3*A*), while TGFβRII^{kd} cells had reduced

SMAD2 phosphorylation (Figure 3*A*). We subsequently determined whether target gene activation was influenced in TGFβ-resistant PAI-1^{kd} cells. We analyzed the expression of the target genes *PAI-1*, *p21^{CIP1}*, and *p15^{INK4b}* in various TGFβ-resistant cell lines by QRT-PCR. We found that, compared to control cells, there was a reduction of the TGFβ target genes *PAI-1*, *p21^{CIP1}*, and *p15^{INK4b}* in TGFβRII^{kd} and SMAD4^{kd} cells (Figure 3*B*). In PAI-1^{kd} cells *PAI-1* levels were strongly reduced, while the induction of cyclin dependent kinase inhibitors *p21^{CIP1}* and *p15^{INK4b}* was unaltered compared to control TGFβ treated cells (Figure 3*B*). uPA over-expressing cell showed comparable *PAI-1*, *p21^{CIP1}* and *p15^{INK4b}* levels to the control (Figure 3*B*), consistent with the notion that uPA is downstream of PAI-1 and not interfering with

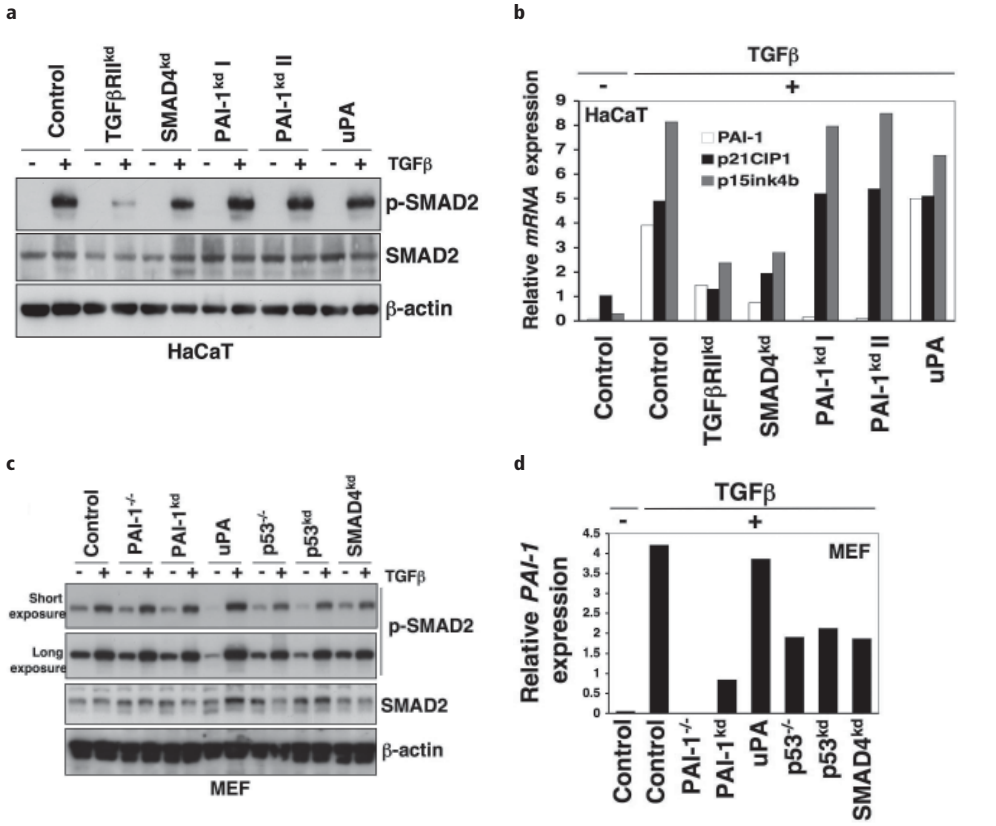


Figure 3 SMAD signaling and target activation in TGFβ-resistant PAI-1^{kd} HaCaT cells and MEFs. *A.* Analysis of SMAD pathway activation in HaCaT cells. Western blot of activated phospho-SMAD2 versus normal SMAD2 in untreated and TGFβ-bypassing cells expressing depicted constructs or a non-functional shRNA control. β-Actin is a loading control. *B.* Analysis of TGFβ-targets *PAI-1*, *p21^{CIP1}*, and *p15^{INK4b}* expression by QRT-PCR, in TGFβ-bypassing HaCaT cells expressing depicted constructs. *C.* Analysis of SMAD-pathway activation in MEFs. Western blot of activated phospho-SMAD2 versus normal SMAD2 in untreated and TGFβ-bypassing cells expressing depicted constructs and non-functional shRNA expressing control cells. β-Actin is a loading control. *D.* mRNA analysis of TGFβ-target *PAI-1* in TGFβ-bypassing MEFs expressing depicted constructs versus a non-functional shRNA expressing and non-treated control.

TGFβ target gene activation. The expression of protein levels of the TGFβ targets *PAI-1* and *p21^{CIP1}* in the various knockdown cells mirrored the mRNA expression by the cells (Supplemental Figure S3). We conclude that loss of PAI-1 in HaCaT cells results in a bypass of the growth inhibitory effect of TGFβ in the presence of a functional SMAD signaling pathway and normal TGFβ target gene activation.

We further analyzed SMAD2 activation in the various TGFβ-resistant MEF cell lines. Ablation of *PAI-1* expression or uPA over-expression did not seem to interfere with SMAD2 phosphorylation in cells treated with TGFβ for ten days, when compared to TGFβ arrested controls (Figure 3*C*), again suggesting that PAI-1 knockdown leaves SMAD activation by TGFβ intact. Using QRT-PCR we measured complete loss and strong reduction of *PAI-1* expression in the *PAI-1$^{-/-}$* and PAI-1^{kd} MEFs, respectively (Figure 3C). The high expression of *PAI-1* in uPA expressing MEFs is again consistent with the notion that uPA is downstream of PAI-1 in TGFβ signaling (Figure 3*D*). We conclude that loss of PAI-1 in MEFs results in a bypass of the growth inhibitory effect of TGFβ in the presence of a functional SMAD signaling pathway and cytostatic target gene activation.

Constitutive PI3K-PKB signaling induces bypass of a TGFβ-induced arrest

Next we sought to determine the downstream mechanism involved in bypass of the TGFβ arrest by *PAI-1* knockdown. Recently we found that loss of PAI-1 causes persistent PI3K-PKB growth factor signaling in aging fibroblasts[15]. Since PKB (AKT) signaling is involved in evasion of the cytostatic activity of TGFβ[18-20], we asked whether in this system loss of *PAI-1* expression might lead to alteration of PKB activation. Indeed, when PAI-1^{kd} HaCaT cells cultured in the presence of 200 pM TGFβ for ten days were assayed for protein levels of active (phopho)-PKB we found that, compared to control-infected cells that arrest with strong reduction in phospho-PKB, both PAI-1^{kd} and uPA over-expressing cells retain activation of PKB as determined by phosphorylation of residue 473 (Figure 4*A*). To address whether activated PI3K-PKB growth factor signaling might also be sufficient to induce a TGFβ-bypass, we stably infected HaCaT cells with retroviral constructs of constitutively active PI3K (caPI3K) or PKB (caPKB), a PTEN knockdown, or control RFP, and tested in a colony formation assay for evasion of the cytostatic activity of TGFβ. PTEN is a potent inhibitor of PKB activation, and loss of this tumor suppressor has been found in many cancers[21]. We found both caPI3K and caPKB activity as well as PTEN^{kd} to be sufficient for bypass of TGFβ arrest (Figure 4*B*). Moreover, activation of PKB was retained when the cells were treated with TGFβ for ten consecutive days (Figure 4*C*). We suggest that loss of *PAI-1* expression results in maintenance of PI3K-PKB signaling and that constitutive activation of PI3K-PKB growth factor signaling is sufficient for evasion of the cytostatic TGFβ response in HaCaT cells.

Discussion

PAI-1 is induced by TGFβ in a variety of cell types, and considered a classical target of TGFβ signaling. So far, however, it has mainly been recognized as being involved in ECM remodeling and tumor-progression[13,14]. We show here that PAI-1 is directly required for the cytostatic activity of TGFβ in human keratinocytes and mouse fibroblasts. Loss of *PAI-1* is as efficient as reduction of canonical TGFβ signaling or SMAD4 levels in bypassing a TGFβ arrest. Apparently, in addition to the effect PAI-1 has on cell motility and invasion, it is also critically involved in TGFβ-dependent proliferation. Our data imply that when the PAI-1:uPA bal-

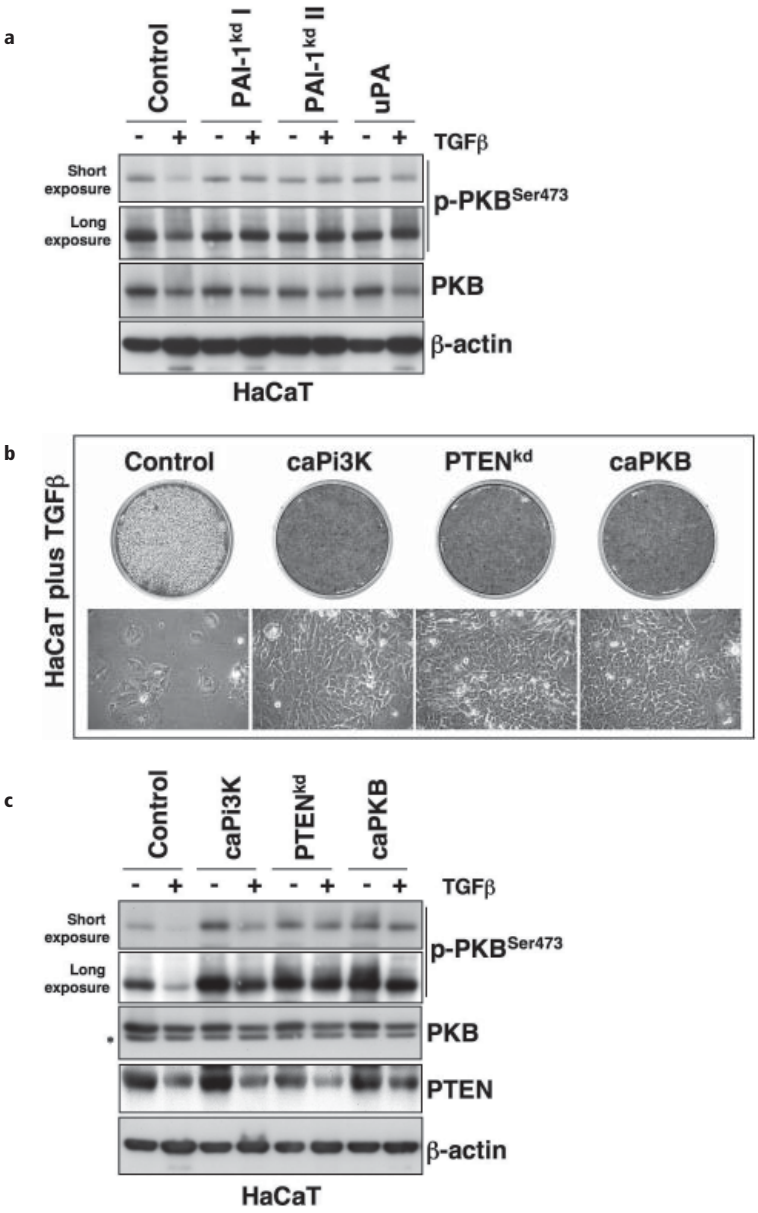


Figure 4 Constitutive PI3K-PKB signaling induces bypass of the cytostatic response to TGFβ in human keratinocytes. *A*. Loss of PAI-1 retains PI3K-PKB signaling in HaCaT cells in the presence of TGFβ. Western blot analysis of activated phosho-PKB versus normal PKB in TGFβ-bypassing cells expressing a non-functional shRNA control or PAI-1^{kd} or uPA over-expressing constructs, respectively. β-Actin is a loading control. *B*. Colony formation assay in HaCaT cells over-expressing indicated constructs treated with TGFβ for 7 days. A non-functional shRNA is used as a negative control. Also shown are phase contrast images of the cells after 7 days in culture in the presence of TGFβ. *C*. Western blot analysis of activated phospho-PKB versus normal PKB and PTEN in TGFβ-bypassing cells expressing a non-functional shRNA control, $PTEN^{kd}$, or constitutively active PI3K or PKB over-expressing construct. Asterix indicates an aspecific background band. β-Actin is a loading control.

ance shifts towards excess uPA, a cell no longer responds to the growth inhibitory effects of TGFβ. We propose that the PAI-1:uPA balance in a cell determines whether the response to TGFβ is anti-proliferative or stimulating tumor progression. Interestingly, in breast cancer, the uPA:PAI-1 ratio is a powerful predictor of disease outcome[22]. PAI-1 may therefore be instrumental for the effects of TGFβ on cancer progression.

We also provide evidence that knockdown of PAI-1 or over-expression of its target uPA enhances PI3K-PKB growth factor signaling in the presence of TGFβ, and that this itself is sufficient for bypass of a TGFβ arrest. Our data support in vivo studies showing that PKB activation makes breast cancer cells less sensitive to the growth-inhibitory effects of TGFβ[23]. TGFβ induction of PAI-1 may, in addition to induction of CDK inhibitors $p21^{CIP1}$ and $p15^{INK4b}$, inhibit cell cycle progression by blocking growth factor dependent CDK activation.

In summary, we suggest that PAI-1 is a critical TGFβ-target required for induction of a cytostatic response in human keratinocytes and mouse embryo fibroblasts, possibly by attenuating PI3K-PKB growth factor signaling.

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Acknowledgements

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Materials and Methods

Antibodies and Vectors Antibodies against PAI-1 (C9), $p21^{CIP1}$ (F5, C19), PKB/Akt1 (C20), PTEN (A2B1) and SMAD2 (S-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti p-PKB (Ser473; #9271) and anti p-SMAD2 (#3101L) were from Cell Signaling (Beverly, MA). β-Actin was from Sigma (Clone AC-74). Anti BrdU antibody (M0744) was from Dako. Mouse and human cDNAs for PAI-1, uPA, PI3K and PKB/AKT have been described[15]. siRNAs in HaCaTs or mouse fibroblasts (MEFs) were produced using the pRETRO-SUPER vector[24]. The 19-mer sequences for PAI-1, SMAD4 and p53 have been described[15,25]. For generation of the human TGFβRII and PTEN knockdown constructs the following 19-mer sequences were used: 5'-GATTCAAGAGTATTCTCAC-3' and 5'-GTGAAGATGACAATCATGT-3', respectively. Controls were retroviral non-functional short hairpin or red fluorescent protein (RFP) vectors.

Cell culture, transfection and retroviral infection Human keratinocyte HaCaT cells, mouse embryo fibroblasts, and Phoenix cells were cultured, transfected, infected or selected as described previously[15]. Human TGFβ (100-B) was from R&D Systems (Minneapolis, MN).

Colony formation assays HaCaT cells were infected with shRNA or cDNA constructs, selected, and 20.000 cells were seeded onto 6 cm plates and stained or counted after 7 or 10 days treatment with 200 pM TGFβ. TGFβ unresponsive and normal HaCaT cells were infected with control vector (RFP) or mouse PAI-1 cDNA, after 72 hours plated under low density (20.000 cells per 6 cm plate), and 1 week later stained or counted. 10.000 or 20.000 young wild-type and post-senescent *PAI-1* and *p53* knockdown and knockout, and uPA over-expressing MEFs were seeded onto 6-well plates and stained after 1 week treatment with no, 10, or 100 pM TGFβ. TGFβ was refreshed every other day.

Growth curves HaCaT cells and MEFs were infected with retroviral shRNA constructs, selected, and 20.000 or 50.000 HaCaT cells or 150.000 MEFs were plated in a 6 cm dish (time = 0 days). Every 4 days cells were counted, and 20.000, 50.000 or 150.000 cells were replated. TGFβ was refreshed every other day. Total cell amounts were displayed as cumulative over time.

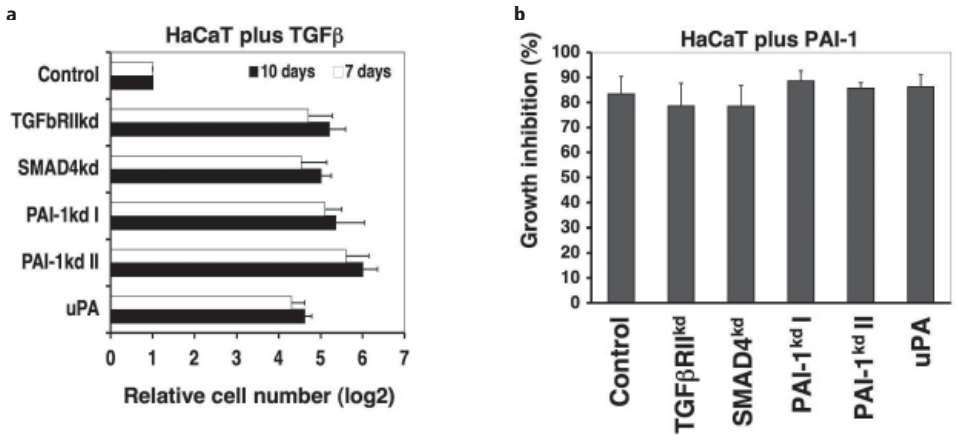
Quantitative real time PCR From untreated or TGFβ-treated HaCaT cells or MEFs RNA was isolated with TRI-Zol® (Invitrogen, Carsbad, CA) according to manufacturer instructions. QRT-PCR was performed on an ABI Prism 7700 with Assays-on-Demand™ (Applied Biosystems) for mouse *PAI-1* and *TBP* as a control housekeeping gene, or for human *PAI-1*, $p21^{CIP1}$ and $p15^{INK4b}$ with *β-Actin* as a control housekeeping gene.

Cell cycle analysis by FACS MEFs were cultured overnight in the absence or presence of 100 pM TGFβ and subsequently incubated with BrdU for 45 minutes. Cells were fixed, permeabilized, and labeled with anti-BrdU and Propidium Iodide (PI).

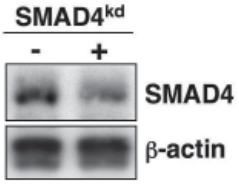
Cell culture Images Phase contrast images were obtained using a Zeiss Axiovert 25 microscope with A-Plan 10x or LD A-plan 20x objectives on a Canon Powershot G3 14x zoom camera.

Western blotting Lysates were from scraped subconfluent plates and complemented with protease inhibitor (Complete, Roche) and phosphatase inhibitor (P2850, Sigma) according to manufactorer instructions. Lysis and analysis was performed as described[15].

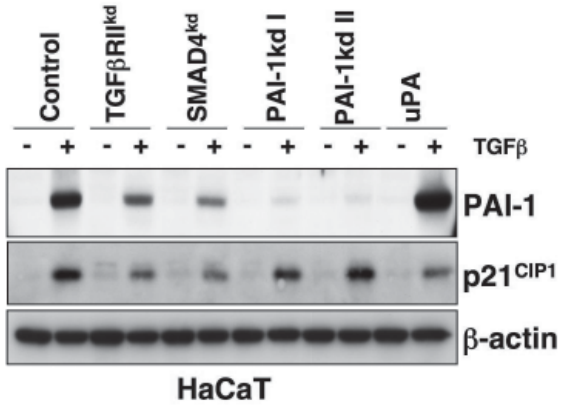
In all experiments representative examples of at least three independent experiments are shown.



Supplemental Figure S1 *A*. Quantification of experiment shown in Figure 1*A*. Shown is amount of HaCaT cells with various indicated constructs after 7 or 10 days TGFβ treatment compared to a non-functional shRNA control. *B*. Quantification of experiment in Figure 1*D*. Shown is reduction of cell-number after retroviral PAI-1 versus empty vector control treatment of various depicted HaCaT cell lines.



Supplemental Figure S2 Western blot analysis of SMAD4 knockdown efficiency in non-functional shRNA expressing control and SMAD4^{kd} MEFs. β-Actin is a loading control.



Supplemental Figure S3 Western blot analysis of TGFβ-targets PAI-1 and p21^{CIP1} in TGFβ-bypassing HaCaT cells expressing depicted constructs. β-Actin is a loading control.

Reason, however, is surely the governing element in such a matter as this; as reason has made the decision concerning the happy life, and concerning virtue and honour also, so she has made the decision with regard to good and evil. For with them the vilest part is allowed to give sentence about the better, so that the senses – dense as they are, and dull, and even more sluggish in man than in the other animals – pass judgment on the Good. Just suppose that one should desire to distinguish tiny objects by the touch rather than by the eyesight! There is no special faculty more subtle and acute than the eye, that would enable us to distinguish between good and evil. You see, therefore, in what ignorance of truth a man spends his days and how abjectly he has overthrown lofty and divine ideals, if he thinks that the sense of touch can pass judgment upon the nature of the Supreme Good and the Supreme Evil! He says: "Just as every science and every art should possess an element that is palpable and capable of being grasped by the senses (their source of origin and growth), even so the happy life derives its foundation and its beginnings from things that are palpable, and from that which falls within the scope of the senses. Surely you admit that the happy life takes its beginnings from things palpable to the senses." But we define as "happy" those things that are in accord with Nature. And that which is in accord with Nature is obvious and can be seen at once – just as easily as that which is complete.

Seneca the Younger, *Letters to Lucilius – Letter 124* (64)

CHAPTER 4

Suppression of the p53-dependent Replicative Senescence Response by Lysophosphatidic Acid Signaling

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Suppression of the p53-dependent Replicative Senescence Response by Lysophosphatidic Acid Signaling

Roderik M. Kortlever[1,2], Thijn R. Brummelkamp[1,2,4], Laurens A. van Meeteren[2,3,5], Wouter H. Moolenaar[2,3] and René Bernards[1,2]

Lysophosphatidic acid (LPA) is a lipid mediator of a large number of biological processes, including wound healing, brain development, vascular remodeling and tumor progression. Its role in tumor progression is probably linked to its ability to induce cell proliferation, migration and survival. In particular, ascites of ovarian cancers is rich in LPA and has been implicated in growth and invasion of ovarian tumor cells. LPA binds to specific G-protein-coupled receptors and thereby activates multiple signal transduction pathways, including those initiated by the small GTPases Ras, Rho, and Rac. We report here a genetic screen with retroviral cDNA expression libraries to identify genes that allow bypass of the p53 dependent replicative senescence response in mouse neuronal cells, conditionally immortalized by a temperature-sensitive mutant of SV40 large T antigen. Using this approach, we identified the lysophosphatidic acid receptor type 2 (LPA_2) and the Rho-specific guanine nucleotide-exchange factor Dbs as potent inducers of senescence-bypass. Enhanced expression of LPA_2 or Dbs also results in senescence-bypass in primary mouse embryo fibroblasts in the presence of wild type p53, in a Rho GTPase dependent manner. Our results reveal a novel and unexpected link between LPA signaling and the p53 tumor suppressive pathway.

Introduction

Tumor suppressor *p53* is often found mutated in cancer and plays a central role in protection against tumorigenesis[1,2]. p53 is a transcription factor that is stabilized and activated by cellular stress, for example in response to DNA damage or oncogene activation[3]. The regulation of p53 itself is complex and involves transcriptional, translational, as well as post-translational modi-

Division of Molecular Carcinogenesis[1], Center for Cancer Genomics[1], Center for Biomedical Genetics[2], and Division of Cellular Biochemistry[3]
The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.
[4] Present address: Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142, USA.
[5] Present address: Uppsala University, Dept. Genetics and Pathology, Uppsala, Sweden.

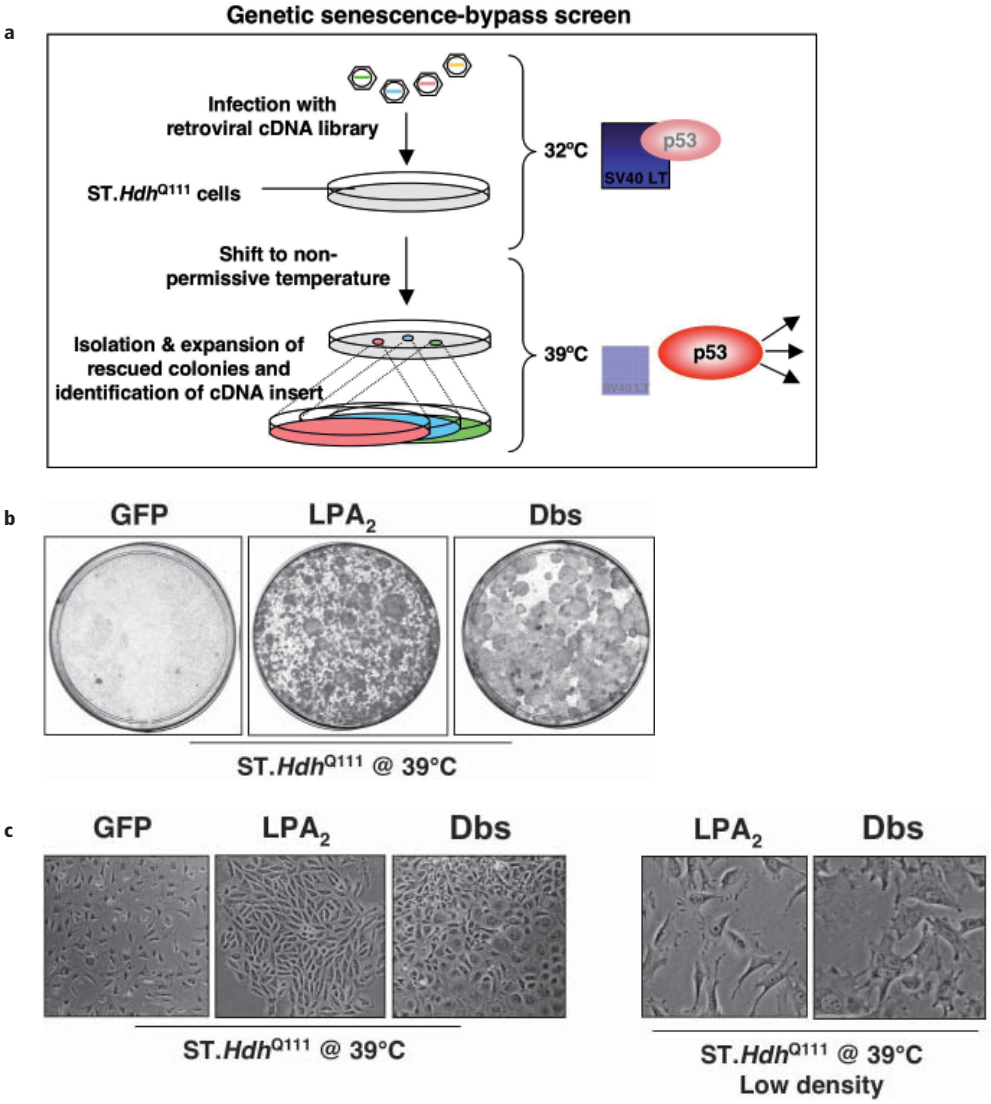

Figure 1 Senescence-bypass screen in conditionally immortalized mouse striatal cells.

(a) Schematic outline of a cDNA library screen for senescence-bypass in conditionally immortalized mouse striatal cells expressing a mutant huntingtin repeat (ST.*Hdh*^{Q111} cells). After infection with a cDNA library at 32°C cells were transferred to the non-permissive temperature of 39°C, where the ST.*Hdh*^{Q111} cells enter a senescence arrest, except when expressing a cDNA that induces a bypass of senescence. These colonies were picked, expanded, and any rescue-inducing cDNAs were subsequently cloned and identified by sequencing.

(b) Colony formation assay for senescence-bypass in ST.*Hdh*^{Q111} cells by recloned LPA_2 and Dbs cDNAs from libraries. GFP is a negative control. Cells were fixed and stained after 10 days.

(c) Phase contrast images taken after 10 days at 39°C of rescued LPA_2 or Dbs over-expressing ST.*Hdh*^{Q111} cells versus senescent cells from (b). A picture of low-density LPA_2- or Dbs- over-expressing ST.*Hdh*^{Q111} cells is added to highlight the phenotypic influence of LPA_2 and Dbs.

fications[4-6]. p53 acts through induction of a wide variety of target genes[4,7,8], which can result in diverse cellular responses, including apoptosis, cell cycle arrest, or senescence[1,6]. Cells deficient for *p53* function will continue to proliferate in the presence of genotoxic stress and thereby accumulate DNA damage[9]. To date, much has been learned about p53 function through study of connective tissue cells like mouse embryo fibroblasts[10,11] (MEFs). When cultured, primary fibroblasts enter into replicative senescence gradually over time, as a result of the stress endured by tissue-culture conditions[3,10,12,13]. This leads to triggering of tumor-suppressive $p19^{ARF}$-p53 signaling, resulting in induction of anti-proliferative p53-targets as *PAI-1* and $p21^{CIP1}$, and a cell cycle arrest[14-17]. Both in human tumors and in mouse models it has recently been confirmed that also *in vivo* senescence is a *bona fide* tumor-suppressive mechanism, and functions as a first barrier to oncogenic transformation[18-21]. In tissue culture, cells may escape senescence by loss of p53 function or its upstream activator $p19^{ARF}$, or by loss of the retinoblastoma family of proteins pRb, p107, and p130 ([15,22-24]), since tumor-suppressive pRb proteins are downstream mediators of p53 in senescence-induction via regulating E2F activity[25,26]. Loss of $p19^{ARF}$ or p53 function can occur through various mechanisms, including mutation, epigenetic gene silencing, repression of $p19^{ARF}$ gene expression (*e.g.* by TBX2 or TBX3), functional inactivation of p53 (e.g. by viral oncogenes as SV40 LT or HPV E6), or by an override downstream in the face of proper tumor-suppressive $p19^{ARF}$-p53 signaling (e.g. loss of p53-targets *PAI-1* or $p21^{CIP1}$) [4,14-16,23,27-29].

Cell cycle progression requires growth factor-dependent RAS and PI3K-PKB signaling to the retinoblastoma family of proteins[17,30]. Mitogenic signaling increases cyclin-dependent kinase (CDK) activity, leading to inactivation by phosphorylation of the pRb-family proteins[15,17]. As a result de-repression of the E2F-family of proteins advances G1 cell cycle progression[25,26,31]. Thus, mitogenic signaling by activation of cyclin-dependent kinases cross-reacts with inhibition of the kinases by $p16^{INK4A}$ and $p19^{ARF}$-p53 signaling on the level of regulation of the pRb G1 cell cycle restriction point.

Among the most abundant serum mitogens is the phospholipid lysophosphatidic acid (LPA). LPA is produced after platelet activation and it promotes the proliferation, migration and survival of many cell types, both normal and malignant (for review see [32-34]). LPA serves as a ligand for at least five distinct G protein-coupled receptors (LPA_1-LPA_5), of which LPA_1, LPA_2 and LPA_3 are the most closely related (formerly known as Edg2, Edg4, and Edg7, respectively (Edg: endothelial differentiation gene))[35]. More recently, new nomenclature has been proposed for these receptors: LPAR1-3, but this is not yet widely used in the field[36]. Increased LPA levels and aberrant LPA-receptor expression have been linked to tumor-progression[34]. Receptor activation by LPA results in the activation of various effectors, including PI3K and the small GTPases Ras, RhoA and Rac, with RhoA activation being particularly prominent[37-39]. Rho activation is mediated by receptor-linked $G\alpha_{12/13}$-proteins signaling to guanine-nucleotide exchange factors (GEFs), which induce translocation of RhoA to the membrane by inducing a GTP-bound state[40,41]. The GEFs of Rho-family GTPases share the Dbl-homology (DH) as a common sequence motif[42]. There is a multitude of GEFs for Rho, including Dbs (Dbl's big sister, also know through its rat orthologue Ost and more recently also referred to as MCF2L)[40,43]. Dbs is capable of transforming NIH3T3 cells (fibroblasts that are deficient for tumor-suppressive $p19^{ARF}$-p53 signaling due to absence of the $p16^{INK4A}/p19^{ARF}$ locus) in a RhoA dependent manner[44,45]. Rho-family pro-

teins and their GEFs regulate the actin cytoskeleton and cell adhesion, and thus are involved in – amongst others – stress fiber formation, cell motility, and cell morphology[41,46]. Their activity is intimately connected to cancer development[40,47].

We used here an unbiased cDNA library screen to identify genes that, when over-expressed, induce a bypass of $p19^{ARF}$-p53 dependent senescence. We describe here an unexpected interaction between LPA receptor signaling and tumor-suppressive p53 activity.

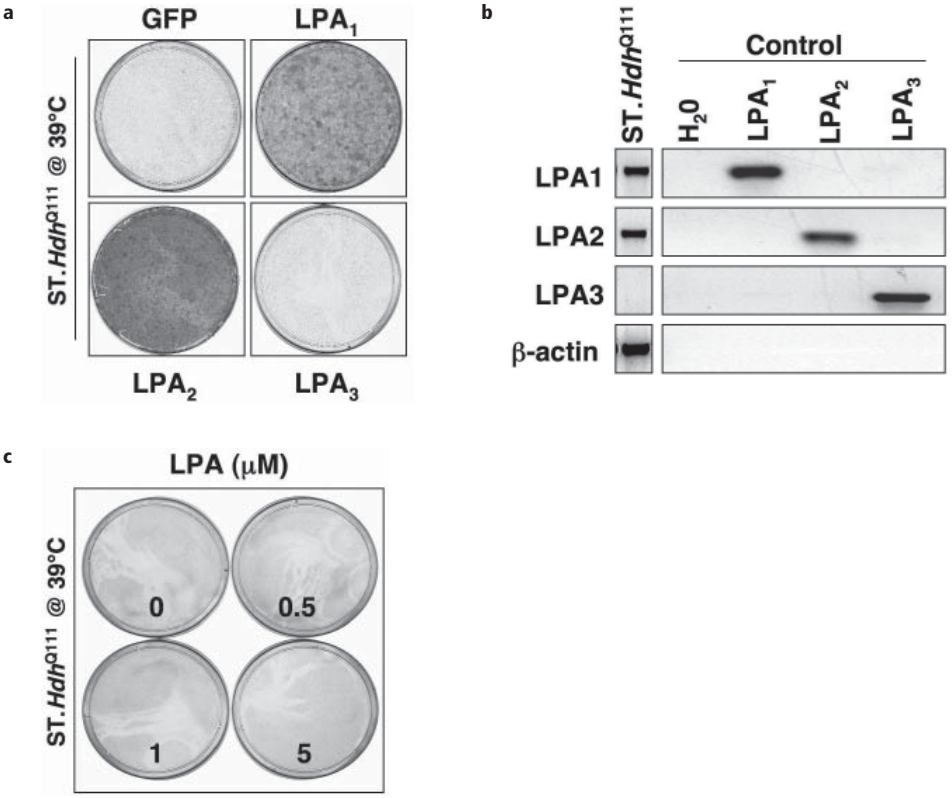

Figure 2 Over-expression of LPA receptor family members but not their ligand induces senescence-bypass.

(a) Colony formation assay for senescence-bypass in ST.*Hdh*^{Q111} cells at 39°C over-expressing LPA_1, LPA_2, or LPA_3, versus a non-functional GFP control. Cells were fixed and stained after 10 days.

(b) Expression of endogenous *LPA_1*, *LPA_2*, or *LPA_3* in normal ST.*Hdh*^{Q111} cells as measured by semi-quantitative RT-PCR. There is no expression of *LPA_3* in normal ST.*Hdh*^{Q111} cells, next to that LPA_3 over-expression does not induce senescence-bypass in these cells.

(c) Analysis of LPA ligand-dependency in senescence bypass in ST.*Hdh*^{Q111} cells. Addition of increasing amounts of LPA to normal media and its influence on senescence-bypass of normal ST.*Hdh*^{Q111} cells at 39°C was measured in a colony formation assay. No proliferation-effect was measured.

Results

To identify cDNAs whose encoded proteins can induce bypass of a p53-dependent senescence arrest, we performed a gain-of-function genetic screen in mouse striatal ST.*Hdh*^{Q111} cells. These mouse neuronal cells harbor a temperature-sensitive mutant of the SV40 Large T (LT) oncogene, which conditionally immortalizes the cells at 32°C, but undergo a robust and uniform senescence arrest after a shift to 39°C, due to inactivation of the LT antigen. A more detailed description of these cells has been published previously[29,48]. We infected the cells at 32°C with retroviral cDNA expression libraries derived from either a polycytemia vera cell line (Osler-Vaquez disease – a chronic myeloproliferative disorder; PCV) or whole human brain (WB), and tested for senescence-bypass following temperature shift of the infected cells to 39°C. We observed no colonies in the GFP-virus infected populations used as a control (data not shown). Infection with the cDNA expression libraries yielded one colony from the PCV library and two independent colonies from the WB library. After expansion of the proliferating senescence bypassing cells we isolated the integrated cDNA inserts by PCR, and subsequently identified their nature by sequencing (Figure 1a).

The inserts we isolated were a full-length cDNAs of G-protein coupled receptor LPA_2 (Edg4) from the PCV library and two independent 5' truncated versions of the GEF Dbs. To confirm that the isolated cDNAs were indeed responsible for the observed bypass of senescence in the original screen we used the isolated and cloned cDNAs from the screen in a second-round assay. We infected ST.*Hdh*^{Q111} cells with pure virus encoding either LPA_2 or Dbs at 32°C and shifted them to 39°C. In a colony formation assay we observed that the cDNAs potently induced a senescence-bypass as compared to a non-functional GFP control (Figure 1b). Moreover, they showed distinct phenotypes when compared to GFP control-infected cells at 39°C (Figure 1c). In case of LPA_2 over-expression there is rounding of ST.*Hdh*^{Q111} cells, which has previously also been observed in other cells of neuronal origin[33,49,50]. In case of Dbs we noticed distinct protrusions at the edge of cells when grown at low density and a transformed-like phenotype when grown at high density (Figure 1c). When we tested the full-length Dbs cDNA, we also observed efficient induction of senescence-bypass (data not shown). We therefore conclude that both LPA_2 and Dbs are able to induce bypass of a p53-dependent senescence-arrest in mouse striatal ST.*Hdh*^{Q111} cells.

As LPA_2 is a member of a family of lysophosphatidic acid (LPA) receptors[35,51], we tested whether other family members could also induce a senescence bypass in ST.*Hdh*^{Q111} cells. We infected ST.*Hdh*^{Q111} cells at 32°C with retroviral vectors harboring the full length cDNAs for LPA_1, LPA_2, and LPA_3, shifted them to 39°C, and tested for senescence-bypass in a colony formation assay. We found that – when compared to a non-functional GFP control – both LPA_1 and LPA_2 were able to induce a senescence-bypass, while over-expression of LPA_3 was not (Figure 2a), even though all three cDNAs were expressed approximately equally (Figure 2b). When we assayed the parental ST.*Hdh*^{Q111} cells for expression of LPA_1, LPA_2, or LPA_3 by RT-PCR we found that these cells naturally express LPA_1 and LPA_2 but not LPA_3 (Figure 2b).

Since we observed that ectopic expression of LPA_2 receptor allows senescence-bypass, we next asked whether triggering LPA receptor signaling by addition of excess ligand could provoke the same response. LPA is a major constituent of serum and is present at concentrations around 1 μM. This suggests that the amount of LPA present in serum is sufficient to saturate endogenous LPA receptors and that addition of even more LPA would not increase signaling and allow senescence bypass. We tested this in a col-

ony formation assay in ST.*Hdh*^{Q111} cells at 39°C following addition of various concentrations of LPA. We observed that besides a change in cell-morphology (data not shown) there was no rescue of senescence up to addition of 5 μM of LPA to the culture medium. As these supra-physiological LPA levels should induce maximal receptor activation, we conclude that the rate-limiting event for senescence bypass in ST.*Hdh*^{Q111} cells is the number of LPA receptors.

The cell system used to identify LPA_2 and Dbs is artificial in that the temperature shift induces a sudden re-activation of p53 and Rb function in the cells. We therefore asked whether we could reproduce the senescence bypass activity of LPA_2 and Dbs in primary mouse embryo fibroblasts (MEFs). Q-PCR analysis revealed that MEFs express both LPA_1 and LPA_2, but not LPA_3, with LPA_1 expression level being 5-10 times higher than LPA_2 (C. Stortelers, L.v.M.

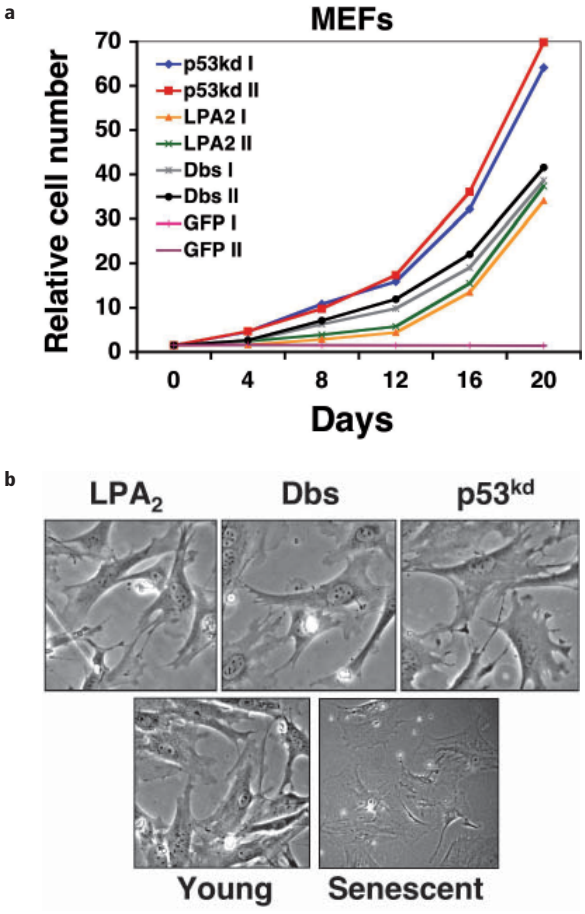

Figure 3 LPA_2 and Dbs induce senescence-bypass in primary mouse embryo fibroblasts.

(a) Growth curve assay for senescence-bypass of LPA_2, Dbs, and $p53^{kd}$ versus non-functional GFP control-infected MEFs. Growth of cells is shown as cumulative over time.

(b) Phase contrast images of rescued LPA_2 or Dbs over-expressing MEFs versus senescent and $p53^{kd}$ controls at day 16.

and W.H.M., unpublished results). We infected two independent populations of late-passage MEFs with either LPA_2 or Dbs retroviral vectors and followed proliferation of these cells over time by making a growth curve. A non-functional GFP was used as a negative control, while a retroviral shRNA against p53 ($p53^{kd}$) was used as a positive control for immortalization [52]. We found that both LPA_2- or Dbs- over-expression immortalized primary MEFs, albeit at a lower efficiency as compared to $p53^{kd}$ (Figure 3a, b). These results indicate that LPA_2 and Dbs are also sufficient for induction of a senescence-bypass in primary mouse fibroblasts.

LPA_2 is a GPCR that signals to Rho GTPases, and Dbs is a GEF for RhoA and Cdc42. We therefore asked whether constitutive RhoA or Cdc42 activity itself is sufficient for senescence bypass. To address this, we used vectors encoding constitutively active mutants of these proteins: $RhoA^{V14}$ and $Cdc42^{V12}$. In a colony formation assay in ST.Hdh^{Q111} cells we found that over-expression of $RhoA^{V14}$ and $Cdc42^{V12}$ indeed mediated senescence bypass (Figure 4a). Furthermore, when tested over a prolonged period of time, we found that enhanced $RhoA^{V14}$ or $Cdc42^{V12}$ expression causes a senescence-bypass in primary MEFs as well (Figure 4b, c). We conclude that constitutive RhoA or Cdc42 signaling is sufficient to bypass senescence.

Our results imply that not only LPA_2 or Dbs over-expression, but also that of their target GTPases RhoA or Cdc42 is sufficient for senescence-bypass in both mouse neuronal and embryo fibroblast cells. This prompted the question of whether the immortalization by LPA_2 or Dbs was Rho-GTPase dependent. Furthermore, we also tested whether the immortal cells were resistant to enhanced p53 signaling. Such experiment would help address whether immortalization by LPA_2 and Dbs is upstream or downstream of p53. When analyzed in a colony formation assay we found that, compared to negative control GFP-protein over-expressing cells, over-expression of a dominant negative mutant of RhoA ($RhoA^{N19}$) completely blocked proliferation in immortalized LPA_2 or Dbs MEFs (Figure 5). The reduction of growth was not due to increased apoptosis (data not shown), and was as effective as the growth-inhibition of the immortal $RhoA^{V14}$ and $Cdc42^{V12}$ cells. MEFs having $p53^{kd}$ or *pRb*$^{-/-}$;*p107*$^{-/-}$;*p130*$^{-/-}$ (TKO: Retinoblastoma-family deficient) MEFs were almost entirely and partially inhibited in their proliferation by $RhoA^{N19}$, respectively (Fig 5). Together, these data suggest that not only the LPA_2 and Dbs cells are heavily dependent on RhoA activity for proliferation, but also $p53^{kd}$, since these cells are also significantly blocked in proliferation. Loss of the pRb-family proteins partially overrides the arrest by $RhoA^{N19}$, suggesting these proteins act downstream of RhoA signaling (Figure 5). We conclude that LPA_2 and Dbs over-expressing cells depend on Rho activity for immortalization. Moreover, we found that not only LPA_2- and Dbs-, but also $RhoA^{V14}$- and $Cdc42^{V12}$-immortalized MEFs were completely refractory to high expression of wild type p53 (Figure 5). TKO control MEFs were also not inhibited by excess p53 expression, in support of the notion that pRb proteins are downstream mediators of a p53-dependent arrest[16,25]. Our results suggest that the senescence-bypass by LPA_2 or Dbs is downstream of p53, but upstream of RhoA.

To analyze further whether the senescence-bypass by over-expression of LPA_2 or Dbs was indeed downstream of p53 we investigated whether the immortalization took place in the presence of normal $p19^{ARF}$-p53 signaling. We first asked whether tumor-suppressive $p19^{ARF}$-p53 signaling was influenced in the immortalized cells, since both the ST.Hdh^{Q111} cells and MEFs are dependent on this signaling pathway for induction of the senescence response[29,48].

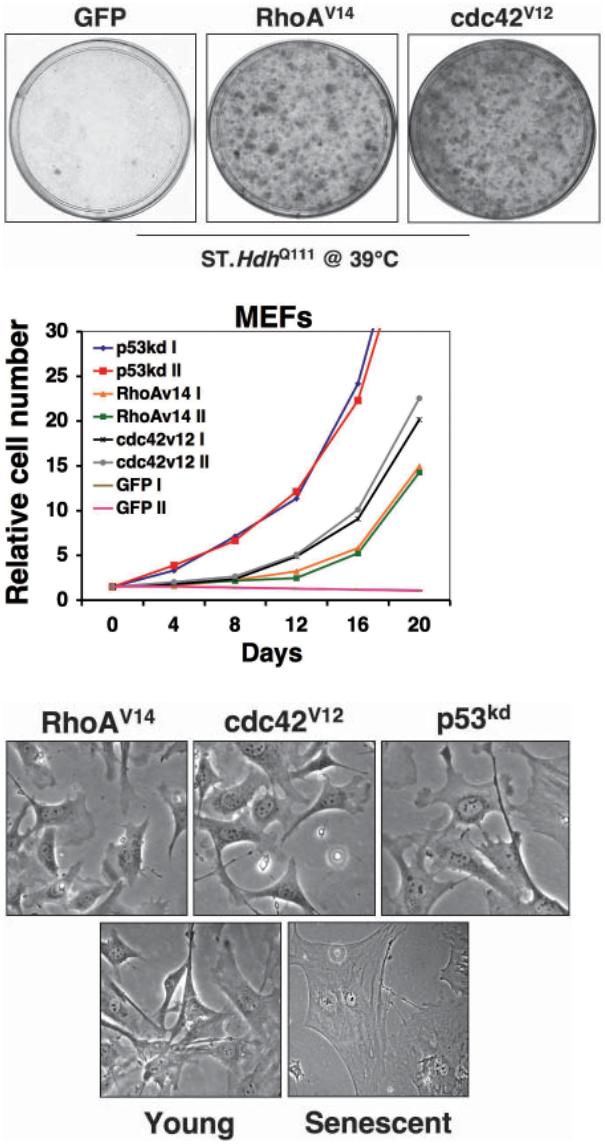

Figure 4 Constitutive Rho-GTPase activity induces senescence-bypass.

(a) Colony formation assay in ST.*Hdh*^{Q111} cells for senescence-bypass after infection with constitutively active RhoA^{V14} and Cdc42^{V12} retroviral cDNA constructs. GFP is a negative control.

(b) Growth curve assay for senescence-bypass of RhoA^{V14}, Cdc42^{V12}, p53^{kd} or non-functional GFP control-infected MEFs. Growth of cells is shown as cumulative over time.

(c) Phase contrast images of rescued RhoA^{V14} or Cdc42^{V12} over-expressing MEFs versus senescent and p53^{kd} controls at day 16.

We analyzed the protein levels of p53 and its downstream target gene *p21*^{CIP1} after the addition of DNA-damaging agent *cis*platin. In normal cycling cells p53 level and activity is low. ST.*Hdh*^{Q111} cells at 32°C exhibited high levels of p53 – in accordance with the notion that SV40 LT blocks and stabilizes p53 at this temperature – which is unchanged after *cis*platin treatment (Figure 6a). The p53 stabilization and p21^{CIP1} induction in the immortalized LPA_2 and Dbs cells after DNA-damage is comparable to the immortal controls, which have wild type p53 (NIH3T3 fibroblasts), indicating that p53 is functional in the immortalized cells (Figure 6a).

Since cells can also bypass senescence by loss of the retinoblastoma family proteins (pRb, p107, p130), and it has been shown that the tumor-suppressive Rb proteins are downstream of p53 in the senescence-induction[25], we investigated whether the activity of E2F, a critical target of Rb family proteins, was altered in the immortalized LPA_2 and Dbs over-expressing cells. We found strong induction of E2F targets cyclin E, p107, E2F1 and PCNA as judged by Western blotting. Their protein levels were distinctly higher than in other immortalized cells (Figure 6b). Note that over-expression of E2F1 itself predominantly induces apoptosis[53], which we only observed with very high expression of LPA_2 (data not shown). We suggest that over-expression of LPA_2 or Dbs in ST.*Hdh*^{Q111} cells leads to senescence-bypass in the presence of functional p19^{ARF}-p53 signaling and is associated with induction of E2F activity.

MEFs are also dependent on a functional p19^{ARF}-p53 pathway and Rb family function for senescence induction [15]. We therefore analyzed in the LPA_2-, Dbs-, RhoA^{V14}- or Cdc42^{V12}-immortalized MEFs whether there was active p19^{ARF}-p53 signaling, as this could further suggest the immortalization was indeed downstream of p53 function. No evidence for loss of p19^{ARF} in the immortalized cells was found (Figure 6c). Compared to p53^{kd} MEFs – which have high p19^{ARF} levels because p53 represses p19^{ARF} expression [6] – there was p19^{ARF} expression in the LPA_2, Dbs, RhoA^{V14} or Cdc42^{V12} over-expressing MEFs, equal to the levels seen in the control infected young and senescent cells (Figure 6c). To measure p53 activity, we further analyzed by western blotting the protein levels of p53 and its target *p21*^{CIP1} in the immortalized cells after the addition of DNA-damaging agent *cis*platin. Compared to young and senescent cells, there was stabilization of p53 and induction of *p21*^{CIP1} in the LPA_2, Dbs, RhoA^{V14} and Cdc42^{V12} over-expressing MEFs, indicating that p53 remained fully functional in these cells (Figure 6c).

We next investigated whether E2F activity was also enhanced in MEFs immortalized by LPA_2, Dbs, RhoA^{V14} and Cdc42^{V12}. When we analyzed the protein levels of E2F-target activation by western blotting, we again observed induction of p107, E2F1 and PCNA when compared to non-cycling senescent cells (Figure 6d). p107 and E2F1 were induced when compared to young cycling cells but equal to levels in cycling p53^{kd} cells, while PCNA levels were comparable between all cycling MEFs (Figure 6d). Apparently, there are differences in specific E2F target activation in the immortalization of MEFs or ST.*Hdh*^{Q111} cells by LPA_2 or Dbs over-expression (see also Figure 6a).

It was recently reported that LPA signaling can lead to downregulation of p53 function by reduction of the nuclear fraction of the protein[54]. We therefore investigated the function and localization of p53 in the immortalized LPA_2 MEFs. Young wild-type MEFs or proliferating and immortalized p53$^{-/-}$ or LPA_2 MEFs were treated with the DNA damaging agent *cis*platin, which leads to activation of p53. Figure 6e shows that p53 was exclusively nuclear in all MEFs and was stabilized and activated in

both wild-type and LPA_2 MEFs, in contrast to $p53^{-/-}$ cells. In accordance there was induction of p53 target $p21^{CIP1}$ in these *cis*platin treated cells (Figure 6e). Quantitative real-time PCR analysis of the same cells showed induction of another bona-fide p53 target, *PAI-1* [55], in both young and immortalized LPA_2 MEFs. The high PAI-1 levels in untreated LPA_2 MEFs compared to untreated wild-type MEFs reflects the p53-dependent induction of PAI-1 in post-senescent cells[16]. It further supports the notion p53 function is unaltered in these cells. In contrast, there was no *PAI-1* induction in $p53^{-/-}$ control MEFs (Figure 6f). Apparently p53 localization and function is not altered in immortalized LPA_2 MEFs. We suggest that, in line with our findings in striatal ST.Hdh^{Q111} cells, LPA_2, Dbs, $RhoA^{V14}$ and $Cdc42^{V12}$ immortalize MEFs downstream of functional $p19^{ARF}$-p53 pathway signaling possibly via induction of E2F activity.

Discussion

We have found in a genetic screen that enhanced expression of the LPA_2 receptor or the Rho-GEF Dbs induces senescence bypass. Furthermore, we report that constitutive Rho-GTPase family signaling by over-expression of active mutants of RhoA or Cdc42 is also sufficient for senescence bypass. LPA_2, Dbs and Rho GTPases mediate bypass of senescence downstream of tumor-suppressive $p19^{ARF}$-p53 signaling, since the immortalized cells still express $p19^{ARF}$ and retain normal p53 function. LPA_2 or Dbs over-expression results in immortalization in the presence of enhanced E2F activity.

So far most links between enhanced LPA_2 expression and cancer are related to later stages of tumorigenesis. For example, a higher rate of lymphatic invasion and metastasis correlates with LPA_2 expression in intestinal-type carcinomas[56], and high LPA_2 expression may to be

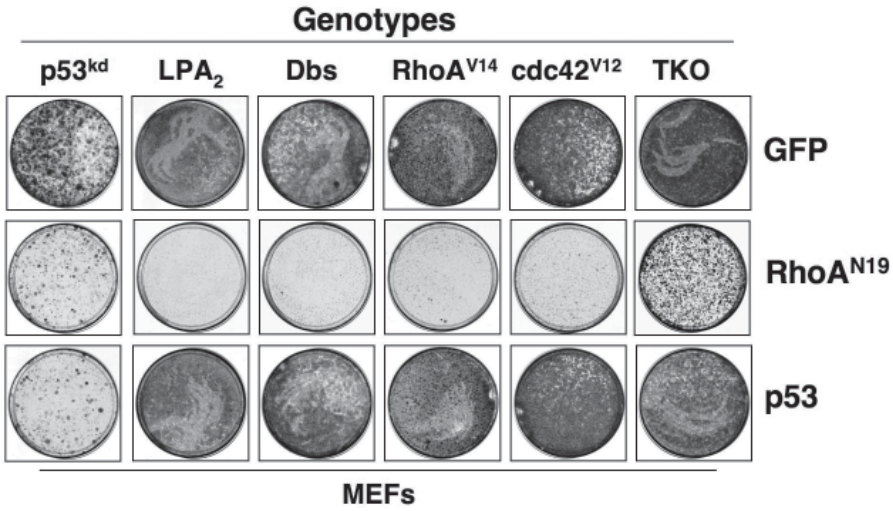

Figure 5 Senescence-bypass by LPA_2 and Dbs is Rho dependent.

Colony formation assay of various depicted immortalized MEFs infected with dominant-negative retroviral cDNA construct for RhoA ($RhoA^{N19}$), p53, or negative control GFP. Growth was in the absence of selection and plates were stained after one week.

involved in thyroid pathogenesis[57]. In ovarian cancer enhanced levels of LPA_2 are frequent, and ascitic fluid from ovarian cancer patients contains elevated levels of LPA[58-60]. In addition, autotaxin, the major LPA-producing enzyme, promotes tumor progression in mouse models and is found overexpressed in various human cancers[34,61-63]. We now find that enhanced LPA_2 activity is also sufficient to bypass p53-dependent senescence. Our findings are consistent with a model in which enhanced LPA signaling can bypass potent tumor-suppressive p53-dependent signaling to mediate escape from replicative senescence. Murph *et al.* recently reported that LPA signaling reduced p53 transcriptional activity and nuclear p53 protein abundance in A549 lung carcinoma cells[54]. In contrast, we find that LPA_2 immortalized cells retain a functional p53 response following treatment with a DNA damaging agent (Figure 6e). Furthermore, Murph *et al.* observe that all three LPA receptors suppress p53 activity, whereas we find that only LPA_1 and LPA_2, but not LPA_3 can bypass p53-dependent senescence. These differences in effect of LPA signaling on p53 may be related to the differences in genetic background used. In our studies we employ non-transformed cells, whereas HepG2 and A549 cells used by Murph *et al*., are cancer cell lines. Irrespective of the precise mechanism(s) involved, both studies highlight an unexpected connection between LPA signaling and p53.

There have been reports of intersection of p53 and Rho GTPase functions. For instance, the migratory effect of p53-loss is PI3K and Rac dependent in 2D and Rho dependent in 3D cultures[64,65], though in 2D culture Rho is normally more associated with invasion[47]. Rho activity is elevated in $p53^{-/-}$ mouse fibroblasts[66] and Cdc42-dependent filopodia formation is interrupted downstream by p53 ([67]). Furthermore, in a non-functional $p19^{ARF}$-p53 background, mitogenic activation of Rho GTPases promotes hyperproliferation and transformation[66,68].

Whether immortalization by LPA_2 or Dbs is critically dependent on actin reorganization or growth factor signaling downstream of RhoA activity needs to be elucidated. The effects that Rho-GTPases have on cell proliferation are so far thought to reflect the crucial roles of anchorage- or adhesion-dependent signals. In the immortalized LPA_2 or Dbs cells E2F activity appears increased as judged by the expression of several of its downstream target genes. Consistent with a possible role for increased E2F signaling in the senescence bypass observed here, it has been shown that increased expression of E2F target genes can mediate escape from senescence[25]. Furthermore, it has been shown previously that LPA induces the expression of urokinase type plasminogen activator (uPA) both *in vitro* and *in vivo*[69,70] and this induction of uPA requires p38MAPK activity [71]. Interestingly, we have recently shown that ectopic expression of uPA or inhibition of the expression of the antagonist of uPA, PAI-1, mediates bypass of senescence[16]. Thus, induction of uPA by LPA signaling may also contribute to the bypass of senescence observed here.

In summary, we describe here a connection between LPA signaling and p53-dependent cell cycle progression. Our findings suggest that LPA_2 and Rho-activity may not only be involved in tumor progression but may also contribute to tumor initiation by regulating p53-dependent senescence.

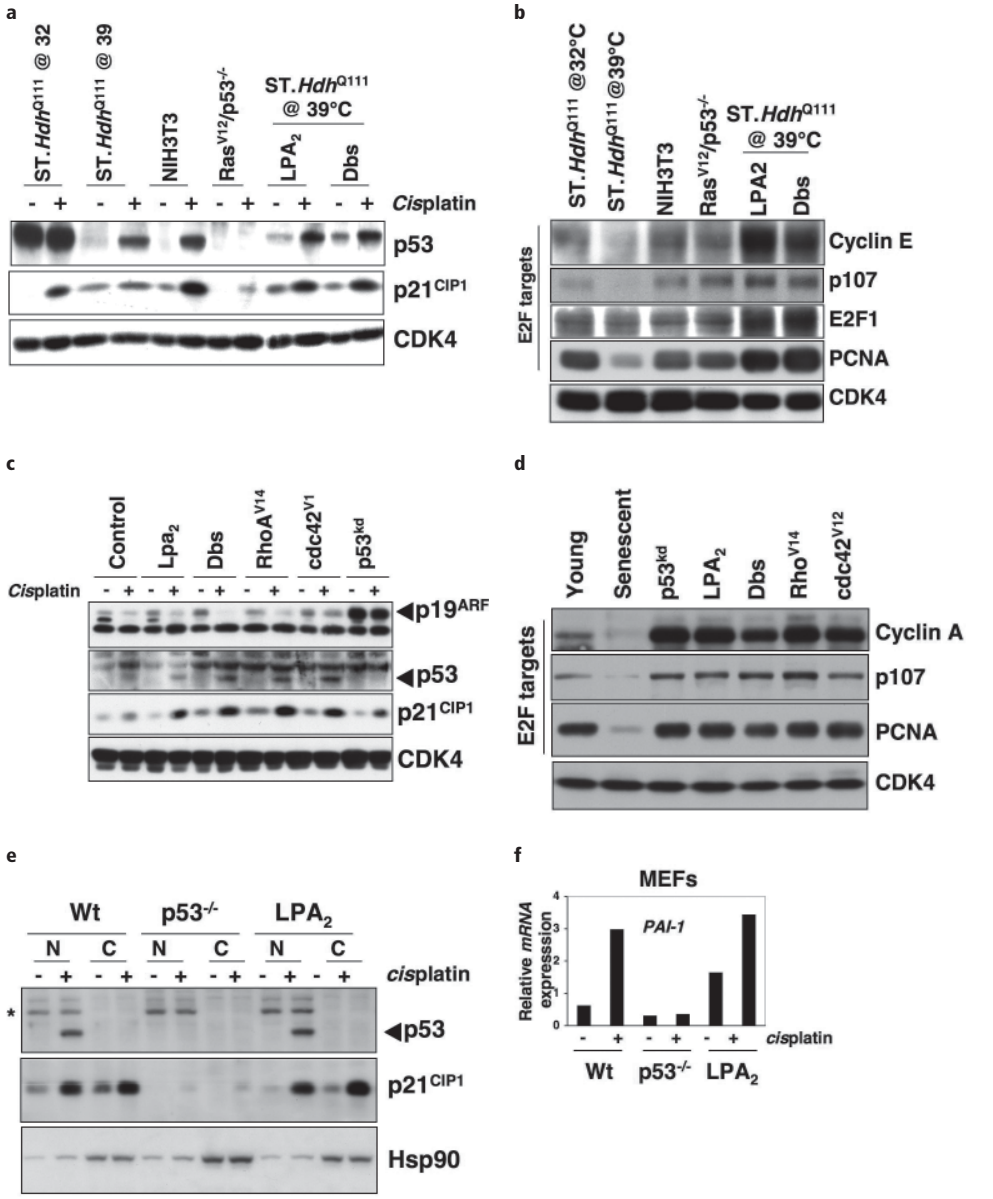

Figure 6 Retention of p53 status and enhanced E2F activity in LPA_2- and Dbs-immortalized ST.Hdh^{Q111} cells and MEFs.
(a) Western blot analysis of senescent normal and LPA_2- and Dbs-immortalized ST.Hdh^{Q111} cells for p53 activation and $p21^{CIP1}$ target induction by *cis*platin-addition, versus cycling NIH3T3 and $Ras^{V12}/p53^{-/-}$ cells. CDK4 is a loading control. Proteins were isolated after 10 days at 39°C. LPA_2- and Dbs-immortalized ST.Hdh^{Q111} cells have normal p53 function.
(b) Western blot analysis of normal and LPA_2- or Dbs-immortalized ST.Hdh^{Q111} cells for E2F targets Cyclin E, p107, E2F1, and PCNA versus cyclin NIH3T3 and $Ras^{V12}/p53^{-/-}$ cells. CDK4 is a loading control. Proteins were isolated after 10 days at 39°C. There is induction of E2F targets in LPA_2- and Dbs-immortalized ST.Hdh^{Q111} mouse striatal cells.

(c) Western blot analysis for $p19^{ARF}$, p53 activation and for its target $p21^{CIP1}$ in young and senescent wild-type MEFs, and LPA_2-, Dbs-, $RhoA^{V14}$-, or $Cdc42^{V12}$-immortalized MEFs after *cis*platin-addition. $p53^{kd}$ is a positive control for loss of p53 function, while CDK4 is a loading control.

(d) Western blot analysis of various normal and immortalized MEFs for E2F targets p107, E2F1, and PCNA versus cycling $p53^{kd}$ cells. CDK4 is a loading control. There is retention of E2F target activation in LPA_2- and Dbs-immortalized cells.

(e) Western blot analysis of nuclear (N) and cytoplasmic (C) protein fractions of young wild-type (wt), or immortal p53 deficient ($p53^{-/-}$) or LPA_2 over-expressing MEFs before (-) and after (+) *cis*platin treatment. Activation of p53 and its target $p21^{CIP1}$ was analyzed by probing with the respective antibody. Hsp90 is a cytoplasmic loading control. Asterix presents a nuclear-specific background band showing equal loading of nuclear fractions.

(f) Quantitative real-time PCR analysis of the induction of *PAI-1* in young wild-type (wt), or immortal p53 deficient ($p53^{-/-}$) or LPA_2 over-expressing MEFs before (-) and after (+) *cis*platin treatment. Control is *GAPDH*.

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Materials and Methods

Antibodies and Vectors For western blotting, antibodies against cyclin E (M20), p107 (C18), E2F1 (C20), PCNA (PC10) and CDK4 (C22) were from Santa Cruz Biotechnology (Santa Cruz, CA), anti p53 (Ab7) from Oncogene Research Products (Boston, MA), and HSP90 (#4874) from Cell Signaling (Beverly, MA). pLZRS-LPA_1 and pLZRS-LPA_3 have been described [72]. Control infections were performed with green fluorescent protein (GFP) vectors.

Cell culture, transfection and retroviral infection Mouse ST.*Hdh*^{Q111} striatal cells, mouse embryo fibroblasts, and Phoenix cells were cultured in DMEM (Gibco) supplemented with 8% heat-inactivated fetal bovine serum (Perbo), 2mM L-Glutamine and penicillin/streptomycin (Gibco). Transfections were performed with the calcium-phosphate precipitation technique. Retroviral supernatants were produced by transfection of Phoenix packaging cells. Viral supernatants were filtered through a 45 μm Millex® HA filter (Millipore, Carrigtwohill, Co. Cork, Ireland), and infections were performed in the presence of 4 μg ml^{-1} polybrene (Sigma). Drug selections in ST.*Hdh*^{Q111} or MEFs were performed with 1 μg ml^{-1} puromycin. ST.*Hdh*^{Q111} mouse striatum cells express a mutant version of the huntingtin protein with an expanded polyglutamine repeat from a knock-in ST.*Hdh*^{Q111} allele and a temperature sensitive mutant of the SV40 T antigen, introduced by retroviral transduction. ST.*Hdh*^{Q111} cells were cultured at 32°C and were shifted to the non-permissive temperature of 39°C when indicated.

Retroviral library screen High-titer retroviral library supernatant derived from human whole brain (Clontech®) or Polycytemia Vera (PCV) cDNA libraries were used to infect 2 × 10^6 ST.*Hdh*^{Q111} cells. Twenty-four hours after infection, cells were plated at a density of 0.8 × 10^5 cells per 10-cm dish and after 48 hours the cells were shifted to the non-permissive temperature of 39°C. Colonies appeared only in the cDNA-library infected populations. These colonies were picked and expanded at 39°C. Retroviral cDNA inserts were PCR-amplified using specific retroviral primers, recloned into pLIB, and identified by sequencing. To analyze whether the recloned cDNA's were responsible for the senescence-bypass, a second round with recloned library cDNA was performed. In case of LPA_2 a full-length cDNA was isolated from the PCV library, in case of Dbs two independent cDNA fragments missing the first 104 amino acids was isolated from the Whole Brain library as the senescence-rescuing constructs. Using specific primers full length versions of LPA_2 (HA-tagged) and Dbs were created, cloned into retroviral pLIB and pBABEpuro vectors, and used in the experiments described in this paper.

Colony formation assays ST.*Hdh*^{Q111} cells were infected with cDNA constructs at 32°C, twenty-four hours after infection cells were selected and plated at a density of 5×10^4 cells per 10-cm dish, and after another 24 hours the cells were shifted to the non-permissive temperature of 39°C. Cells were stained after 10 days at 39°C. For all colony formations representative examples of at least three independent experiments are shown. High purity grade LPA was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). p53^{kd}-, LPA_2-, Dbs-, RhoA^{V14}-, Cdc42^{V12}- and TKO-immortalized MEFs were infected with GFP, RhoA^{N19} or p53 virus, and 50.000 cells were plated on a 10-cm dish. Plates were stained after one week.

Growth curves Late passage MEFs were infected with retroviral cDNA constructs, selected, and 150.000 MEFs were plated in a 6 cm dish (time = 0 days). Every 4 days cells were counted, and 150.000 cells were re-plated. Total cell amounts in all growth curves were displayed as cumulative over time. For all growth-curves representative examples of at least three independent experiments are shown.

Real-time PCR everse transcriptase and PCR reaction were performed following standard procedures. First strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen) and oligo(dT) primers. $LPA_{1,2,3}$-specific 20-mers were designed to recognize human LPA receptors, lacking any significant similarity with other LPA receptors.

Quantitative real-time PCR rom untreated and *cis*platin treated young wild-type, p53$^{-/-}$ and LPA_2 MEFs total RNA was isolated with TRI-Zol® (Invitrogen, Carlsbad, CA) according to manufacturer instructions. QRT-PCR was performed on an ABI Prism 7700 with Assays-on-Demand™ (Applied Biosystems) for mouse *PAI-1* and *GAPDH* as a control housekeeping gene

Cell culture Images Images were obtained using a Zeiss Axiovert 25 microscope with A-Plan 10x or LD A-plan 20x objectives on a Canon Powershot G3 14x zoom camera.

Protein fractioning From untreated and *cis*-platin treated young wild-type, p53$^{-/-}$ and LPA_2 MEFs nuclear and cytoplasmic protein fractions were isolated with Nuclear and Cytoplasmic Extraction kit NE-PER® (Pierce Biotechnology Inc., Rockford, IL) according to manufacturer instructions.

Western blotting Selected cells were lysed in RIPA buffer (50 mM Tris pH 8; 150 mM NaCl; 1% NP40; 0,5% DOC; 0,1% SDS). 40 or 80 micrograms of protein was separated on 8-12% SDS-polyacryamide gel by electrophoresis and transferred to polyvinylidine difluoride membranes (Millipore). Western blots were probed with the indicated antibodies. For all western blots representative examples of at least two independent experiments are shown.

I am faring to visit the limits of the all-nurturing earth, and Oceanus, from whom the gods are sprung, and mother Tethys, even them that lovingly nursed me and cherished me in their halls. Them am I faring to visit, and will loose for them their endless strife, since now for long time's apace they hold aloof one from the other from the marriage-bed and from love, for that wrath hath fallen upon their hearts. And my horses stand at the foot of many-fountained Ida, my horses that shall bear me both over the solid land and the waters of the sea. But now it is because of thee that I am come hither down from Olympus, lest haply thou mightest wax wroth with me hereafter, if without a word I depart to the house of deep-flowing Oceanus.

Homer, *Iliad – 14* (600-700 BC)
Translation by Robert Fagles (1990)

CHAPTER 5

General Discussion

Adapted from Cell Cycle 2006 Dec; 5 (23): 2697-2703

General Discussion

We have uncovered critical interactions between extra-cellular factors PAI-1, uPA, LPA and tumor-suppressors p53 and TGFβ. I first will discuss here what led us to think that PAI-1 may be involved in senescence, and provide data that the effect of PAI-1 loss may be cell non-autonomous as well. Second, I will integrate our discoveries on extra-cellular proteins PAI-1 and uPA, the tumor suppressors p53 and TGFβ, and the phospholipid LPA with their known role in the fibroblast wound healing response. Finally, I will speculate on how this may help us in better understanding the analogies between the wound healing response and the tumor-stroma interaction during cancer progression.

Studying senescence

We used a biased approach in tracking suspects that may be causally involved in escape of two types of proliferation arrest: senescence and the cytostatic response to TGFβ. We zoomed in on a candidate that is highly stimulated by classic tumor-suppressors, and which has previously ascribed critical roles in angiogenesis and invasion. As escape of homeostasis is governed by extra-cellular proteolytic activity that somehow lays the foundation for angiogenesis, proliferation, and migration, our molecule of interest may unexpectedly show off its versatility in multi-step neoplastic transformation.

p53 and PAI-1

To investigate the relationship between serum stimulation, wound healing and senescence, we focused our attention on Plasminogen Activator Inhibitor 1 (PAI-1). Our interest in this gene was sparked by several observations: (i) 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 up-regulation of the activity of both over time, with senescent cells and closing wounds having the highest levels of p53 and PAI-1[1-4], (ii) PAI-1 is a senescence marker in fibroblasts, both *in vivo* and *in vitro*[2,5], (iii) p53 is critically involved in the senescence response of fibroblasts[6], and progressively up-regulates PAI-1 expression during prolonged culturing in serum[2], (iv) The serum response profile of lung fibroblasts includes induction of urokinase type plasminogen activator (uPA)[7] and among the activated genes in the poor prognosis wound-like signature are plasminogen and the uPA receptor[8,9], both of which function downstream of PAI-1 and are antagonized by PAI-1 ([10,11]), (v) 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

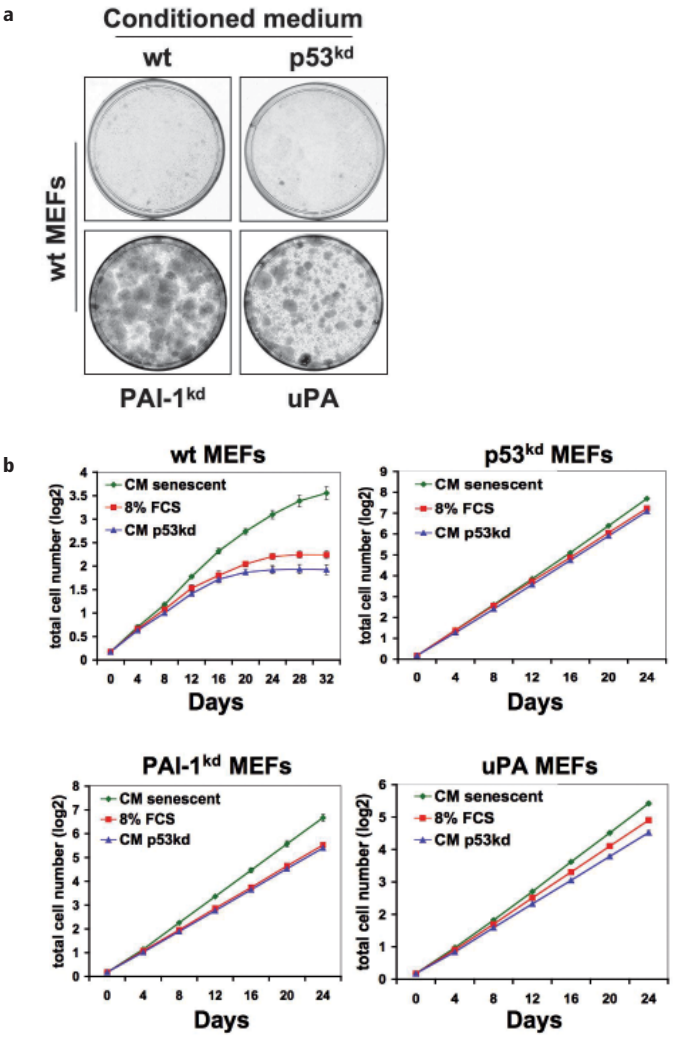

Figure 1 Loss of p53 or PAI-1 expression has a cell-autonomous or non-cell-autonomous effect on immortalization.

(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-1^{kd}, or uPA over-expressing 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. After time is 0 days 150.000 cells were transferred to a 6 cm dish, every 4 days cells were counted, and 150.000 cells replated. Growth is depicted as cumulative over time. The mean (+/- sd) per triplicate of 3 different media additions per genotype is shown.

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[12,13], (vi) Mice bearing a hyperactive allele of tumor suppressor p53 have premature aging and reduced wound healing phenotypes[14]. 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 (see Chapter 2) and, in turn, whether heterotypic signaling might dictate the proliferative response (see below).

PAI-1 and senescence

In Chapter 2 we show that down-regulation of *PAI-1* induces immortalization through bypass of senescence in a cell-autonomous fashion in both mouse and human fibroblasts[15]. 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 over-expression 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 over-expression of uPA leads to paracrine signaling with growth-stimulatory effects towards wild type fibroblasts. We transferred conditioned medium from immortal PAI-1 knockdown cells (PAI-1^{kd}) or uPA over-expressing MEFs to pre-senescent wild type MEFs and studied the effect of these conditioned media on the proliferation of the pre-senescent MEFs. Figure 1a shows that the transfer of conditioned medium from immortal PAI-1^{kd} (kd: knockdown) or uPA over-expressing MEFs to pre-senescent 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 (Figure 1a). This suggests that PAI-1^{kd} or uPA over-expression induces a senescence-bypass that is not strictly cell autonomous. p53^{kd} MEFs have higher amounts of *PAI-1* compared to PAI-1^{kd} cells[15] 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 (Figure 1a). We note that cell-nonautonomous effects on proliferation caused by p53 have been observed before[16,17].

This brought us to the second question, namely 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 over-expressing 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 over-expressing 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 (Figure 1b). This is most likely explained by the findings of Krtolica *et al.*, who showed that senescent fibroblasts secrete mitogenic factors[18]. 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[15]. 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 (R. Kortlever and R. Bernards, 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 over-expression of uPA in aging 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 may play a role in the induction of senescence in a paracrine fashion.

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*. Aging fibroblasts, besides up-regulating *PAI-1*, $p21^{CIP1}$ and $p16^{INK4A}$, down-regulate PI3K-PKB signaling, and the activity of this particular mitogenic signaling pathway may be a central intermediary in the induction of senescence by p53 ([15,19]). Down-regulation of p53 or PAI-1, or over-expression of uPA may result 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[15].

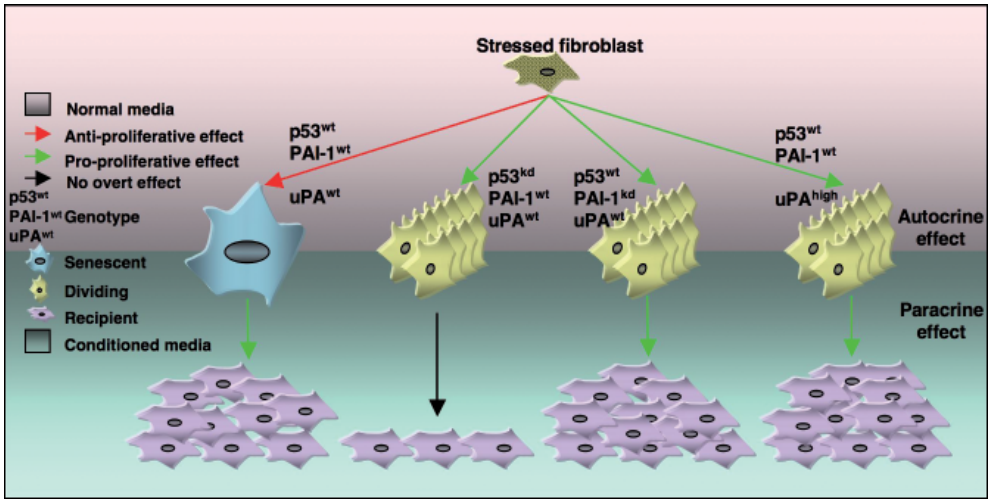

Figure 2 Model of cell-autonomous and cell-nonautonomous effects on fibroblast immortalization after loss of PAI-1 or uPA over-expression.

We describe that cells become unresponsive to mitogenic signaling by antagonizing uPA through p53-mediated up-regulation 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 Figure 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 aging from unlimited proliferation but at the same time such cells stimulate surrounding cells to proliferate[20]. 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.

Wound-healing and cancer

By studying escape of a G1 arrest via both a biased and unbiased manner we have uncovered that genes previously associated with inflammation, angiogenesis and wound healing are directly involved in p53-dependent senescence-bypass or bypass of the cytostatic activity of TGFβ. Since wound healing has parallels with cancer progression (see for example Figure 4 in Chapter 1), we now discuss how our results may aid to a better understanding of tumorigenesis. We describe which roles the factors investigated in this thesis play in the physiological and pathophysiological responses to wounding (with an emphasis on fibroblasts), and the analogies with cancer progression.

p53, TGFβ, PAI-1, uPA and LPA in wounding

The sequence of events in wounding is as follows[21-32], and consists roughly of three phases: First, after the blood components spill into the site of injury, platelets come into contact with ECM-associated proteins. This triggers release of clotting factors, cytokines and growth factors as, for example, LPA, interleukins, TGFβ, PAI-1 and uPA. Subsequently, the connective tissue and endothelial cells respond by further release of cytokines, growth factors and proteases as PAI-1, uPA, TGFβ, EGF, FGFs, and others. The effect of the cytokines is vascular dilation and capillary leakage. Additionally, there is formation of oxygen free radicals and p53 activation. Thus, haemostasis happens in the presence of various growth and differentiation signals emanating from TGFβ and the uPA-PAI-1 equilibrium. Second, after this haemostatic phase, inflammation is induced by the entry of neutrophils, macrophages and leukocytes into the wound, and production of more growth factors and proteases in order to activate the surrounding fibroblasts. As a result, the fibroblasts migrate and proliferate into the wound and deposit a rigid ECM structure mainly consisting of fibrin and collagen, a process dependent on uPA and PAI-1 activity. During this granulation tissue formation the migrating fibroblasts differentiate into myofibroblasts, which is TGFβ dependent. The ECM scaffold acts as a platform for attraction of keratinocytes and endothelial cells to re-establish normal blood-fed skin tissue. The orchestration of the proliferative phase occurs through the cytokines produced by macrophages, fibroblasts and keratinocytes as well as from the direct interaction of these cells with the ECM. Finally, and third, the remodeling phase consists of cross-linking the collagen and formation of scar tissue. Thus, the gradient of growth factors and their inhibitors in healing of a wound shifts over time from a growth-stimulating to a growth-inhibiting one, or, in other

words, from proliferation and migration to differentiation. The regulation of genes after tissue injury *in vivo* is paralleled by the transcriptional 'wound signature' response of fibroblasts to the soluble fraction of clotted blood, or serum[33]. This 'wound signature' is a powerful predictor of metastasis and death in diverse types of primary tumors[9].

p53, TGFβ, PAI-1, uPA and LPA in disease

In vivo, it has been shown that aberrant TGFβ, LPA and PAI-1 signaling are involved in linking haemostasis with cancer. Excessive blood clotting to the extent that it leads to neoplastic transformation of liver cells is dependent on PAI-1 and COX2 ([34]). Interestingly, the role of PAI-1 and COX2 in oncogene-induced hypercoagulation was determined after they both were found highly upregulated in *cMET*–overexpressing haemorrhagic liver cells. Hyperactivation of blood clotting is associated with an increased metastatic tendency[35]. The induction of coagulation may therefore be an important effector mechanism of the invasive-growth program that provides a fibrin mesh to support cell proliferation and a trail for motility and invasion[34,36]. Additionally, both LPA and TGFβ signaling stimulate vascular leak, a process dependent on proliferation and detachment of endothelial cells, main producers of PAI-1. Vascular leak after lung injury, a TGFβ dependent process, is abrogated in $LPA_1^{-/-}$ mice, leading to pulmonary fibrosis, or excessive deposition of collagens and fibrin[37]. Furthermore, high PAI-1 levels are associated with vascular diseases as thrombosis and artherosclerosis[38]. Apparently, the timely coordinated activation of proteases and their inhibitors is disrupted and this prohibits or prematurely induces the next healing state.

Additionally, TGFβ gene transfer to the lungs of mice results in induction of pulmonary fibrosis after bleomycin treatment[39-41]. In line with this it has been observed that loss of PAI-1 reduces, while over-expression of PAI-1 enhances lung fibrosis in mice[42]. In human high expression of PAI-1 is associated with various fibrotic diseases as IPF (idiopatic pulmonary fibrosis), sarcoidosis, asbestosis, or cystic fibrosis. This coincides with a reduced fibrinolysis and excessive fibrin deposition, a process resulting in accumulation of ECM and reduction of the plasticity of cells[42]. Fibrosis is further characterized by intense proliferation and accumulation of myofibroblasts that synthesize ECM and pro-inflammatory cytokines[43,44]. So both vascular leak and fibrosis seem to be induced by disproportional TGFβ, PAI-1 or LPA activity. Constitutive PAI-1 expression may therefore instigate either hyperactive acute healing or premature induction of late phase wound healing and overstimulation of associated attracted (myo)fibroblasts. Indeed, in cutaneous wound healing there is consistent and phasic TGFβ and PAI-1 induction, respectively, overlapping in the acute and late healing phase[2,4]. Another impaired wound healing response is formation of chronic ulcers, which are due to an impaired response of fibroblasts to TGFβ, since this molecule is trapped to pericapillary polymerized fibrin molecules[28]. Chronic venous ulcers are marked by excessive infiltration of MMP-secreting neutrophils and reactive oxygen species[26,45]. Interestingly, fibroblasts from venous ulcers have been shown to be unresponsive to growth-inhibiting TGFβ activity due to low TGFβRII levels[46]. On the other hand, another study has shown that fibroblasts from venous ulcers cultured *in vitro* exhibit signs of premature senescence, with elevated levels of TGFβ, PAI-1 and plasmin[47]. Therefore, the loss of an arrest in fibroblasts with low PAI-1 or high uPA levels in response to p53 or TGFβ we find *in vitro* mirrors accelerated or delayed wound healing phenotypes observed in mice knockout or transgene for these molecules, respec-

tively. Additionally, their expression levels are also instructive in other pathological disorders as fibrosis and ulcers, as found both in patients and mouse models.

LPA signaling is linked to uPA production. LPA concentrations equivalent to those present in ovarian cancer ascitis fluid stimulate uPA production and secretion in ovarian cancer cell lines[48]. A possible mechanism may be via Ras-Raf dependent activation of NF-κB (a transcription factor and central mediator of pro-inflammatory cues) ([49]). uPA is a metastasis-promoting enzyme and its concentration in ascites is inversely correlated with ovarian cancer prognosis[48]. Next to uPA, LPA is able to induce proliferation and migration of fibroblasts intro an open wound, suggesting their recruitment by an inflammatory environment. Additionally, just as uPA and PAI-1, LPA is not only involved in fibrosis but also in obesity and artherosclerosis[50-54]. We now find that enhanced LPA signaling is powerful enough to induce escape of a p53 arrest, possibly via RhoA activation. RhoA is involved in actin rearrangements and smooth muscle activation[55-57], and apparently capable of immortalizing fibroblasts when constitutively active (see Chapter 4), hence stimulating both their proliferative and contractile capabilities. Perhaps this indicates that this molecule may be involved in more than only tumor progression.

However, *in vivo* the role of LPA in wound healing seems complicated. Topical application of LPA in a full-thickness wound of rat skin induces contraction but only modestly proliferation of fibroblasts[21] yet it promotes neo-epithelial thickness, while macrophages are the primary responsive cells[27]. Furthermore, induction of proliferation and migration of epithelial cells seems TGFβ independent[22], and ectopic LPA inhibits proliferation of keratinocytes, perhaps by collaborating with SMAD transducers of TGFβ signaling[58]. Nevertheless, high expression of autotaxin, the precursor for LPA, is associated with cancer progression[59] and with invasiveness of breast cancer cells *in vitro* ([60]).

Since both PAI-1 and LPA_2 knockouts lack an obvious phenotype, this implies redundancy or compensatory effects and this may only become apparent under pathophysiological conditions as inflammation and wound healing. While LPA seems to be more of a recruiting factor, both uPA and PAI-1 directly determine the proliferation and role of fibroblasts in wound closure. Furthermore, the latter are also direct targets in tumor-inhibiting signaling by p53 and TGFβ (this thesis). This raises multiple questions, among which whether these newly discovered connections may provide novel insights in cancer progression which can be exploited to the benefit of cancer patients.

Tumor-Stroma interactions in cancer

Can our findings help us to improve understanding of the parallels between wound healing and metastasis? PAI-1 and uPA appear to be central players in senescence and wound healing, and their activation is regulated by inflammation. This and other insights may provide a basis to understand the interactions between stromal tissue and cancer cells.

uPA and PAI-1 in (stromal) fibroblasts

uPA/PAI-1 deregulation may influence intratumoral heterotypic signaling and alter the tumor microenvironment. Such a model is tempting since: (i) it is becoming increasingly evident that tumors rely on interactions with stromal tissue, of which the principal cells are fibroblasts[61-63], (ii) the transcriptional response of fibroblasts to serum is also seen in the tumors of breast cancer patients having poor prognosis[9], (iii) PAI-1 and uPA are highly expressed by leading edge fibroblasts and myofibroblasts in breast cancer stromal tissue[5,64,65] and (iv) PAI-1, uPA and PAR have been prospectively validated as

markers of poor prognosis for breast cancer[66,67]. Interestingly, while both high uPA or high PAI-1 levels are individual markers for bad prognosis, high expression of both is an even better prognostic factor[67,68]. uPA and PAI-1 are amongst the most reliable markers whose clinical values were confirmed in LOE 1 (Level of Evidence 1) studies by the European Organization for Research and Treatment of Cancer (EORTC) ([69]), and can therefore be considered for routine assessment of prognosis in new patients with breast cancer. Interestingly, they may also be new well-needed markers for axillary node negative patients[68,70]. uPA is already a prognostic factor for breast cancer subtype groups as axillary node positive, pre- and post-menopausal, and ER-positive patients[71].

The role and regulation of uPA and PAI-1 during metastasis is complex and sometimes conflicting. 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[72,73]. Furthermore, both uPA transgenic and uPA knockout mice show reduced metastasis in syngeneic or xenograft mammary tumor models[74-76], 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[73], 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 vascularization[72,77].

In normal human breast tissue levels of uPA and PAI-1 are very low[71]. In pre-malignant lesions or ductal carcinoma *in situ* (DCIS) tumor epithelial cells stain consistently but weakly positive for uPA, uPAR and PAI-1 ([78]), while PAI-1 is mainly found in high-grade DCIS myoepithelial cells[79]. In invasive breast carcinoma, uPA expression is mainly in the stromal cells as (myo)fibroblasts and macrophages[71]. The data from patients and mice 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[67,80].

TGFβ and PAI-1 in (stromal) fibroblasts

TGFβ plays a critical role in cancer progression as well. One of the target genes of SMAD activity that is potently induced by TGFβ in keratinocytes, fibroblasts, epithelial, and endothelial cells is *PAI-1* ([81,82]). Similar to PAI-1, TGFβ is involved in extra-cellular matrix homeostasis and angiogenesis, and associated with cancer progression[10,83]. Besides this, TGFβ receptor knockout studies have established its important anti-inflammatory functions[84,85].

In Chapter 3 we describe that a TGFβ-induced G1 arrest is also PAI-1 dependent. PAI-1 is thus not only a critical target of p53 in proliferation control but also of TGFβ, and its activation by TGFβ is dependent on p53[86]. As for PAI-1, TGFβ is involved in fibrosis, wound healing and metastasis and capable of inducing ECM-remodeling by regulating plasmin and MMP activity. Elevated PAI-1 levels are associated with high TGFβ expression and ECM deposition under various pathologic conditions, indicating a critical role for PAI-1 in TGFβ-induced fibrogenesis[42]. Furthermore, TGFβ activity is directly involved in the conversion of a fibroblast to a myofibroblast[44,87], cells that appear to be responsible for PAI-1 secretion at the leading edge of stromal tissue in cancer invasion[64,65]. There is *in vivo* evidence to suggest that stromal 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. Other studies imply that molecules like TNFα and HGF, which themselves are regulated by uPA-PAI-1 function, are involved in this cell-nonautonomous effect of loss of tumor-suppressive TGFβ activity in breast stromal fibroblasts[89]. Similarly, though only *in vitro*, we find that the PAI-1 levels secreted by fibroblasts can determine a cell-nonautonomous immortalizing effect (see above). In pancreatic cancer cells, expression of an inhibitory soluble form of the TGFβRII leads to reduced cancer growth, decreased neoangiogenesis, and decreased expression of both PAI-1 and uPA ([90,91]). Apparently, there are analogies between signaling pathways downstream of the uPA-PAI-1 system and TGFβ, whose spatial organization drives cell-cell communication[31,61,92].

An integrative view

As elaborated upon above, TGFβ, PAI-1 and LPA stimulate contraction and/or myofibroblast conversion, but we now find that uPA and LPA also stimulate proliferation of a fibroblast to a degree that they are no longer growth-inhibited by TGFβ or p53 signaling. How should we interpret this? Perhaps in disease excessive TGFβ, PAI-1 or LPA activity leads to a more contractile (myo)fibroblast phenotype and fibrin deposition, but overactive LPA or uPA signaling may at the same time also induce proliferation and ECM remodeling to such extent that the differentiation state of wound healing, namely p53 and TGFβ dependent arrest and subsequently necrosis, is bypassed. Obviously, this may well be a very local effect highly influenced by the characters of surrounding cells. Translated to stromal tissue: in case of a uPA-PAI-1 ratio in favor of uPA activity, this may lead to both endocrine and paracrine-induced escape of growth-inhibition and pro-invasive and contractile fibroblast behavior. The intracellular signaling pathways responsible for this may be Rho-dependent smooth muscle activation and PI3K-PKB growth factor signaling dependent progression of the cell cycle. Additionally, disturbance of the equilibrium in favor of uPA activity may also prompt cells to evade the growth inhibitory signals of TGFβ, perhaps hinting towards a role for PAI-1 and uPA in the shift from a growth inhibitory to a growth-promoting role of TGFβ in cancer progression. Collectively, as a result, the growth-inhibition escaping and pro-migratory stromal fibroblasts may locally stimulate adjacent tumor cells, leading to infiltration of surrounding tissue and progression of disease. However, this needs further investigation and validation in complex models.

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Ontsnappen aan Groeiremming in Kanker

NEDERLANDSTALIGE SAMENVATTING VOOR DE LEEK

Ontsnappen aan Groeiremming in Kanker

(SAMENVATTING VOOR DE LEEK)

Kanker is een ziekte van de genen. Een gen is een code voor een eiwit; de uiteindelijke bouwblokken van een cel die verantwoordelijk zijn voor de communicatie zowel binnen als buiten de cel of tussen cellen onderling. Normaalgesproken voeren de cellen in ons lichaam een specifieke functie uit of zijn in ruste. Dit wordt geïnstrueerd vanuit de genen: het DNA oftewel erfelijk materiaal. Mutaties in het DNA kunnen echter leiden tot verstoring van het natuurlijk evenwicht waardoor de cel een eigen leven gaat leiden. Hierdoor ontstaat een afwijkend gedrag dat kan resulteren in kanker of uitzaaiingen daarvan, in het laatste geval vrijwel zeker leidend tot de dood. Om dit tegen te gaan hebben de cellen in ons lichaam remmingsmechanismen die ongeremde celgroei – een essentieel onderdeel van het vormen van een kanker – blokkeren. De blauwdrukken van die remmingsmechanismen liggen gecodeerd als genen in de kern van een cel. Deze remmingsmechanismen worden geactiveerd door bijvoorbeeld DNA-schade of hyperactivatie van groeistimulerende genen. Genen die beschermen tegen kanker worden tumor-suppressors genoemd en genen die kanker bevorderen worden oncogenen genoemd. Verlies van de functie van een tumor-suppressor veroorzaakt groei, terwijl hyperactivatie van de functie van een oncogen ook groei bevordert. Om kanker te voorkomen is het noodzakelijk de functie van een tumor-suppressor te behouden en activatie van oncogenen tegen te gaan. In dit proefschrift wordt nieuw inzicht gegeven in hoe enkele van de in kanker meest gemuteerde tumor-suppressors groei remmen.

Verrassenderwijs hebben we ontdekt dat groeiremming plaatsvindt door genen te activeren waarvan tot nu toe werd vermoed dat deze alleen betrokken waren bij communicatie tussen cellen onderling via de buitenkant van de cel, oftewel de extracellulaire ruimte. Deze extracellulaire ruimte is een depot met groeifactoren die klaar staan om groei te bevorderen en bijeen gehouden wordt door een netwerk van structurele eiwitten tussen de cellen in. Normaalgesproken wordt er een evenwicht tussen groeiactivators en groeiremmers in stand gehouden die er voor zorgt dat er alleen gedoseerd groeifactoren vrijkomen in de extracellulaire ruimte. In dit proefschrift wordt beschreven dat tumor-suppressors groei kunnen reguleren door de extracellulaire ruimte direct te beïnvloeden. We concluderen dat functieverlies van een tumor-suppressor het equivalent is van

hyperactivatie van een cascade aan groeifactoren, uiteindelijk beiden leidend tot het negeren van groeiremming van een cel.

Bestudeerd zijn enkele genen wiens functie verloren is of ondermijnd wordt in de meeste zo niet alle kankers, namelijk p53 en TGFβ. p53 is een gen dat na activatie cellen 'tot rust' maant. p53 kan ontregelde cellen beschermen tegen ongeremde celdeling door ze aan te zetten tot zelfdoding (apoptose) of het kan cellen dwingen tot een permanente groeistop (senescence). Bij ruim vijftig procent van alle tumoren zitten er fouten in p53. Het defecte gen kan zijn remmende werking niet meer uitoefenen en een potentiële kankercel is geboren. In Hoofdstuk 2 wordt beschreven dat p53 plasminogen activator inhibitor-1 (PAI-1) gebruikt in senescence. Na activatie van p53 door stress is recrutering en activatie van PAI-1 vereist voor p53 om senescence te kunnen induceren. PAI-1 is een soort poortwachter die bepaald hoeveel groeifactoren in de extracellulaire ruimte vrij komen door het extracellulaire netwerk van structurele eiwitten te beïnvloeden. Indien dit niet op de juiste manier gebeurd zal een cel gevoeliger worden voor groeifactoren. Hierdoor zal het senescence-signaal genegeerd worden door hyperactivatie van groeifactor signalering en het bevorderen van de activiteit van een eiwit genaamd Cycline D1. p53 heeft PAI-1 nodig om groeifactoren te remmen en zodoende Cycline D1 uit de kern van de cel te weren, aangezien dit leidt tot senescence. Indien PAI-1 niet aanwezig is, zal p53 activiteit Cyline D1 niet uit de kern kunnen weren en zal de cel zijn groeiremming verliezen. Er vindt dan ontsnapping aan groeiremming plaats en dit bevordert kanker. Van PAI-1 was het bekend dat het een rol speelt bij het uitzaaien van kankercellen. Het eiwit heeft eveneens een voorspellende waarde in de vraag welke borstkankerpatiënten een grote kans hebben op het ontwikkelen van uitzaaiingen. We veronderstellen dat PAI-1 ook direct betrokken is bij de eerste ontwikkeling in kanker, namelijk het remmen van celgroei. Mogelijk is het zelfs zo dat verlies van PAI-1 activiteit in één cel andere cellen in de omgeving ook tot groei kan stimuleren, waardoor het omzeilen van tumor-suppressor activiteit besmettelijk wordt (zie Hoofdstuk 5).

In hoofdstuk 3 wordt beschreven dat PAI-1 ook essentieel is voor de anti-tumor ofwel cytostatische werking van TGFβ. TGFβ is een molecuul dat z'n rol vertolkt in de extracellulaire ruimte en betrokken is bij diverse aspecten van het gedrag van cellen zoals deling en migratie. Het mysterieuze aan TGFβ is dat het vroeg tijdens tumorontwikkeling celdeling tegengaat maar later tijdens ontwikkeling van de tumor juist deling en tumorvorming bevordert. Ergens in het begin van de ontwikkeling van een tumor raakt TGFβ het vermogen kwijt groei te remmen, en de balans slaat door naar TGFβ-gevoeligheid die juist groei bevordert. TGFβ kan vele genen reguleren, waaronder PAI-1. We hebben ontdekt dat PAI-1 nodig is voor TGFβ om een cel te kunnen laten stoppen met groei, oftewel cytostase te bevorderen. Net als bij het omzeilen van p53 zorgt PAI-1 verlies in cellen ervoor dat groeifactor signalering geactiveerd wordt. Dit veroorzaakt ongevoeligheid voor de groeiremmende activiteit van TGFβ.

We concluderen dat twee belangrijke tumorsuppressors, p53 and TGFβ, beiden hetzelfde principe gebruiken om groei te remmen, namelijk door signalering van groeifactoren te blokkeren. Deze recente inzichten maken duidelijk dat PAI-1 betrokken lijkt bij zowel ongeremde celgroei als bij het ontstaan van uitzaaiingen. Mogelijk is PAI-1 een schakel tussen tumorinitiatie en -progressie en kunnen de nieuwe bevindingen een duidelijker beeld verschaffen omtrent hoe een tumor zich daadwerkelijk ontwikkelt.

Groeifactor signalering die leidt tot het negeren van p53 activiteit kan ook plaatsvinden

via hyperactivatie van eiwitreceptoren. Dit zijn eiwitten die in de buitenkant van een cel, het celmembraan, verankert liggen en betrokken zijn bij het doorgeven van signalen van de buitenkant van de cel naar de kern, daar waar de genen gereguleerd worden. In een experiment waarin er gekeken werd naar de activiteit van een miljoen genen tegelijk kwam naar voren dat hyperactivatie van de lysophosphatidic acid receptor 2 (LPA_2) p53-afhankelijke senescence voorkomt (Hoofdstuk 4). In plaats van een directe invloed op activatie van groeifactor signalering, suggereren we dat LPA_2 cellen laat ontsnappen aan groeiremming door het skelet van een cel dusdanig van binnenuit in z'n structuur te beïnvloeden waardoor direct of indirect via groeifactoren de groei gestimuleerd wordt.

De factoren die p53- en TGFβ-afhankelijke groeiremming kunnen beïnvloeden zijn bekend uit het onderzoek naar het helen van een wond. Kanker wordt ook wel vergeleken met een wond die niet heelt, maar er is maar een rudimentair beeld van de processen en moleculen die hierbij betrokken zijn. De mechanismen zijn grofweg bekend, zoals een inflammatoire respons en celspecifiek gedrag, maar welke moleculen centraal zijn is nauwelijks bekend. Er is eerder aangetoond dat PAI-1 en LPA causaal betrokken zijn bij processen die metastasering bevorderen en een rol vertolken in de biologische mechanismen van het helen van een wond. In dit proefschrift wordt beschreven dat PAI-1 en LPA ook noodzakelijk kunnen zijn in het begin van kanker, namelijk voor het ontsnappen aan groeiremming. Door dit onderzoek zijn nieuwe schakels tussen processen binnenin de cel en de buitenkant blootgelegd. Tumor-suppressors als p53 of TGFβ rekruteren bepaalde moleculen in de extracellulaire ruimte die vervolgens op een dominante manier groeiremming kunnen bevorderen.

Mogelijkerwijs kunnen de nieuw ontdekte parallellen tussen wondheling en p53- en TGFβ-afhankelijke ongeremde celdeling een beter inzicht verzorgen omtrent welke processen en moleculen noodzakelijk verbonden zijn met de progressie van kanker.

Curriculum Vitae

Roderik Marco Kortlever was born around lunchtime on Monday 24th of August, 1970. After obtaining his HAVO highschool diploma at the Samenwerkingsschool in Waddinxveen, he went on to study for research technician at the Hoger Laboratorium Onderwijs in Delft, part of the Hogeschool Rotterdam en Omstreken (now Rotterdam University). He did his internship at Gist-Brocades (now DSM) in Delft, where he worked on the isolation and identification of a toxin from Saccharomyces Kluyveri for use as a biopreservative. Roderik received his Bachelor in 1993. Subsequently he went to the University of Leiden to study Chemistry from 1993 to 1999. During his specialization he did an internship on the role of the pseudoknot in the tRNA-like 3' end of Turnip Yellow Mosaic Virus RNA in the *in vitro* initiation of the (-) strand synthesis in the Genexpress group of Prof. Dr. Kees Pleij, under supervision of PhD student Birgit Deiman. He then became a research technician at the Molecular Carcinogenesis division of the Netherlands Cancer Institute in Amsterdam. He worked under supervision of PhD student Thijn Brummelkamp in the group of Prof. Dr. René Bernards, where he learned about the cell cycle. After two years he started his own PhD research and unraveled novel genetic networks of interaction between some of the most mutated genes in cancer. This research is described in this thesis, and was performed between January 2002 and January 2008. Roderik lives in San Francisco, USA, and is a post-doctoral researcher in the group of Prof. Dr. Gerard Evan. He studies mouse models of cancer at the Pathology Department of the University of California, San Francisco.

Roderik Marco Kortlever werd geboren rond lunchtijd op Maandag 24 Augustus 1970. Na z'n HAVO diploma verkregen te hebben op de Samenwerkingsschool in Waddinxveen vetrok hij naar Delft om voor laborant te studeren aan het Hoger Laboratorium Onderwijs, onderdeel van de Hogeschool Rotterdam & Omstreken (nu Rotterdam Universiteit). Hij doorliep z'n stage bij Gist-brocades (nu DSM) in Delft, alwaar hij werkte aan de isolatie en identificatie van een toxine uit Saccharomyces Kluyveri die gebruikt kan worden als conserveermiddel. Roderik behaalde zijn HLO diploma in 1993. Vervolgens studeerde hij Scheikunde aan de Universiteit van Leiden tussen 1993 en 1999. Gedurende z'n specialisatie deed hij onderzoek naar de rol van de pseudoknoop in het tRNA-achtige 3' uiteinde van het Turnip (Koolraap) Yellow Mosaic Virus RNA in de *in vitro* initiatie van de (-) streng synthese. De stage werd uitgevoerd in de Genexpress groep van Prof. Dr. Kees Pleij onder begeleiding van promovenda Birgit Deiman. Hieropvolgend werd hij laborant op de Moleculaire Carcinogenese afdeling van het Nederlands Kanker Instituut. Hij werkte onder begeleiding van promovendus Thijn Brummelkamp in de groep van Prof. Dr. René Bernards en leerde hier over de celcyclus. Na twee jaar startte hij z'n eigen promotieonderzoek en ontrafelde nieuwe genetische netwerken van interactie tussen sommige van de meest gemuteerde genen in kanker. Dat onderzoek is beschreven in dit proefschrift en is uitgevoerd tussen Januari 2002 en Januari 2008. Roderik woont in San Francisco, Verenigde Staten en is post-doctoraal onderzoeker in de groep van Prof. Dr. Gerard Evan. Hij bestudeert muize kanker modellen op de Pathologie afdeling van de Universiteit van California, San Francisco.

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