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Research Article

PAI-1 Expression Is Required for HDACi-Induced Proliferative Arrest in *ras*-Transformed Renal Epithelial Cells

Stephen P. Higgins, Craig E. Higgins, and Paul J. Higgins

Center for Cell Biology and Cancer Research, Albany Medical College (MC-165), Albany, NY 12208, USA

Correspondence should be addressed to Paul J. Higgins, higginp@mail.amc.edu

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Malignant transformation of mammalian cells with *ras* family oncogenes results in dramatic changes in cellular architecture and growth traits. The generation of flat revertants of v-K-*ras*-transformed renal cells by exposure to the histone deacetylase inhibitor sodium butyrate (NaB) was previously found to be dependent on transcriptional activation of the PAI-1 (SERPINE1) gene (encoding the type-1 inhibitor of urokinase and tissue-type plasminogen activators). NaB-initiated PAI-1 expression preceded induced cell spreading and entry into G_1 arrest. To assess the relevance of PAI-1 induction to growth arrest in this cell system more critically, two complementary approaches were used. The addition of a stable, long half-life, recombinant PAI-1 mutant to PAI-1-deficient v-K-*ras*-/c-Ha-*ras*-transformants or to PAI-1 functionally null, NaB-resistant, 4HH cells (engineered by antisense knockdown of PAI-1 mRNA transcripts) resulted in marked cytostasis in the absence of NaB. The transfection of *ras*-transformed cells with the Rc/CMVPAI expression construct, moreover, significantly elevated constitutive PAI-1 synthesis (10- to 20-fold) with a concomitant reduction in proliferative rate. These data suggest that high-level PAI-1 expression suppresses growth of chronic *ras*-oncogene transformed cells and is likely a major cytostatic effector of NaB exposure.

1. Introduction

Histone acetyltransferases (HATs) transfer acetyl groups from acetyl CoA to specific lysine residues in the amino terminal histone "tails" to form ε-N-acetyl lysine promoting an "open" or relaxed chromatin structure. Several transcriptional coactivators, including CBP/p300 and SRC, have intrinsic HAT activity [1, 2]. Histone deacetylases (HDACs), in contrast, catalyze the removal of acetyl groups on target lysines [3, 4] creating a condensed, transcriptionally repressed, chromatin organization [5]. Of the various HDAC inhibitors (HDACi), several exhibit more or less specificity for individual members of the four classes (I-VI) of human HDACs [6, 7].

A major mode of action of HDACi (i.e., the transcription-dependent mechanism) [5] affects gene reprogramming as a consequence of HDACi type, concentration, and duration of exposure [8, 9]. Recent estimates place the number of HDACi-impacted genes at 2–10% of the total expressed repertoire, several of which negatively regulate cell cycle progression [10–13] such as $p21^{WAF1/CIP1}$ and plasminogen activator inhibitor type-1 (PAI-1; SERPINE1) [14–21]. PAI-1 is particularly relevant in this context as this SERPIN complexes with both urokinase (uPA) and tissue-type (tPA) plasminogen activators to limit pericellular plasmin generation effectively attenuating uPA-/plasmin-dependent growth factor activation and cellular proliferative responses [22, 23]. PAI-1, in fact, is both necessary and sufficient for p53-dependent growth arrest [23–26] and required for TGF-β1-mediated antiproliferative effects in human keratinocytes and mouse embryo fibroblasts [27]. Activated *ras* or *raf* oncogenes trigger the initiation of a senescence-like growth arrest program, with induction of PAI-1, in several cell types [28–31]. At least some cells transformed as a consequence of chronic oncogenic *ras* expression, and that escape *ras*-induced senescence can also undergo proliferative arrest upon exposure to certain HDACi (e.g., sodium butyrate; NaB) with concomitant high-level PAI-1 induction [16, 17, 32]. It is not known, however, if HDACi-associated growth inhibition of immortalized *ras*-transformants, like

entrance of normal cells into replicative senescence [23], also requires PAI-1-induction.

This paper details the involvement of PAI-1 expression during HDACi-induced growth restriction in several well-characterized v-Ki-*ras* and Ha-*ras* $^{val-12}$-transformed epithelial cell lines [15, 16]. NaB was selected for this study as this HDACi is a potent stimulator of PAI-1 expression [14, 20] and cytostasis [16] in *ras*-transformed renal cells. NaB-mediated growth inhibition was not evident in PAI-1 knockdown (i.e., anti-sense) cells, but the HDACi-dependent proliferative block could be rescued by vector-driven PAI-1 overexpression.

2. Materials and Methods

2.1. Culture Conditions and Engineered Cells. The various *ras*-transformed renal epithelial cell lines used in this study [15, 16] grow in serum-free DMEM (at least over the short time frame used in this study; 3–5 days) facilitating assessments of the proliferation-modulating effects of NaB (1–10 mM) and exogenous PAI-1 (0.02–100 nM stable mutant 14-1B, $t_{1/2}$ = 145 hours; N150H, K154T, Q319L, M354I) [33] in both the presence and absence of FBS. The derivation of the PAI-1 functionally null knockdown (PAI-1^{KD}) 4HH cell line by transfection of a 2.6 kb rat PAI-1 *Eco*R1/*Hin*dIII cDNA fragment (representing nucleotides −118 to +2572) cloned in anti-sense orientation (Rc/CMVIAP) has been described [34, 35]. v-*ras*-transformed cells were also transfected with the Rc/CMVPAI sense vector to initiate high-level PAI-1 expression in the absence of NaB or with the empty Rc/CMV construct [32]. Coupled *in vitro* transcription/translation assay confirmed that a full-length immunoreactive PAI-1 protein was synthesized using the Rc/CMVPAI vector as a template [35]. In some cases, Rc/CMVPAI transfectants were selected with G418 [32]. Cloning strategy and cell line derivation are detailed in the text. c-Ha-*ras* oncogene-expressing human HaCaT II-4 keratinocytes were described previously [33, 36] as were the PAI-1-deficient and reconstituted renal cell lines [35].

2.2. Northern Blotting. Cytoplasmic RNA was separated by electrophoresis on denaturing 1% agarose/2.2 M formaldehyde gels, transferred to nitrocellulose and blots hybridized with a ^{32}P-labeled EcoRI-HindIII fragment of rat PAI-1 cDNA (specific activity 1-2 × 10^8 cpm/μg DNA) for 48 hr at 4°C. The recombinant pBluescript (SK(-) phagemid pRPAISS1-3, containing a 3.0-kb EcoRI/SstII-flanked cDNA insert encoding PAI-1, was used for isolation of the pRPAImr1-4 probe used for hybridization. Briefly, pRPAISS1-3 was digested with EcoRI/Hind III at 37°C for 1 hr and fragments separated in 1% agarose gels. After staining with ethidium bromide, bands representing the PAI-1 cDNA insert were excised and electroeluted. This insert fragment (pRPAImr1-4) was labeled with ^{32}P-dCTP by random priming. Following hybridization, membranes were washed sequentially for 20 minutes each in 2x SSC/0.1% SDS (twice) and then in 1x SSC/0.1% SDS, all at 55°C.

2.3. Extraction of Metabolically Labeled Cells and Gel Electrophoresis. Growth media (in 35-mm diameter cultures) were aspirated, cells washed twice with HBSS and 1 mL of labeling medium (FBS- and methionine-free RPMI 1640 medium containing 50 μCi ^{35}S-methionine (specific activity = 1100 Ci/mmol) added to each culture. At the end of a 6 hr labeling period, the substrate adherent-enriched (SAP) cellular fraction was collected, clarified at 13,000 ×g, and solubilized in lysis buffer (9.8 M urea, 2% Nonidet P-40, 2% ampholytes, and 100 mm dithiothreitol) [37]. 1-D gel separations were as detailed previously [38]. For 2-D gel electrophoresis, 50,000 cpm ^{35}S-methionine-labeled protein were loaded onto prerun 1.5 mm diameter tube gels (9.1 M urea, 2% Nonidet P-40, 6% pH 5–7 ampholytes, 1.2% pH 3–10 ampholytes, 4% acrylamide/bisacrylamide for isoelectric focusing (IEF) for 18 hr prior to separation on SDS-10% acrylamide slab gels [39]. Individual protein spots were mapped and quantitated with a Bio-Image Investigator 2-D Electrophoresis Analysis system interfaced to a SUN SPARC workstation [40].

3. Results

3.1. PAI-1 Induction in v-ras-Transformed Renal Cells upon Exposure to the HDACi NaB. Limited expression profiling previously indicated that PAI-1 was among the most abundant of the NaB-upregulated genes in *ras*-transformed renal epithelial cells [14, 15] consistent with microarray and bioinformatic analyses of genetic networks responsive to NaB in colonic epithelial cells [20]. 1-D electrophoresis (Figure 1(a)), northern blotting (Figure 1(b)), and 2-D proteomic mapping (Figure 1(c)), moreover, confirmed a significant and rather selective PAI-1 induction in NaB-stimulated v-*ras*-transformants (involving both the 50-kD and mature 52-kD glycosylated PAI-1 species), using 1-D/2-D mobility and immunochemical identification criteria established previously [16, 38, 41], relative to nondetectable PAI-1 levels in control v-*ras* populations. PAI-1 upregulation correlated with a prominent NaB-associated G_1 arrest increased cell size (Figure 2) and concentration-dependent proliferative inhibition resulting in a 63% (Figure 3(a)) and 48% (Figure 3(b)) decrease in population density in serum-free and 10% serum-supplemented medium, respectively (summarized in Figure 4). Collectively, these findings (Figures 1 and 4) are consistent with the conclusion that v-*ras*-transformants that escape *ras* oncogene-initiated cellular senescence [29, 42, 43] are essentially PAI-1 null.

3.2. Growth Arrest in ras-Transformants Is Restored by Exogenous Exposure to a Long Half-Life PAI-1 Mutant or by Vector-Driven Reconstitution of PAI-1 Expression. Since targeted suppression of PAI-1 leads to bypass of both replicative senescence and TGF-β-induced growth arrest [23, 27], it was important to determine if exogenously-delivered PAI-1 could similarly regulate the proliferative response of PAI-1-deficient *ras* transformants in the absence of NaB. The addition of a long half-life recombinant PAI-1 mutant (PAI-1 14-1B) effectively suppressed growth of v-*ras*-transformed

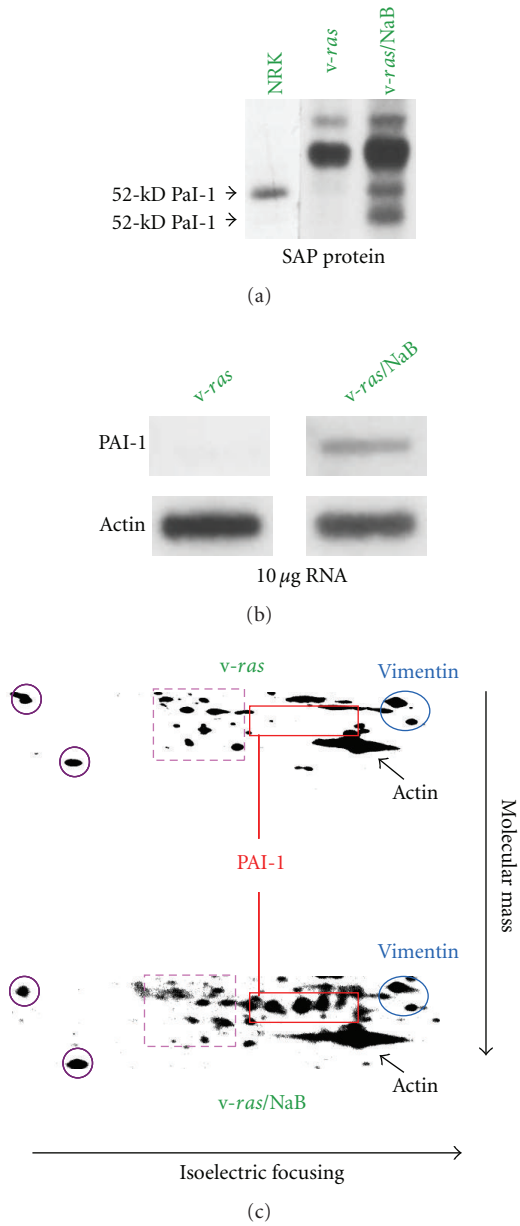

Figure 1: Electrophoresis of the ^{35}S-methionine-labeled saponin-resistant (SAP) protein fraction of v-*ras*-transformed renal cells and their NaB-treated counterparts indicated that PAI-1 (both the 50-kD and fully glycosylated 52-kD species) were expressed in NaB revertants but not in untreated cells (a). PAI-1 from normal kidney cells (NRK) served as a marker. The band above PAI-1 in v-*ras* cells (at 62-kD) is the heavily-glycosylated forms of osteopontin (a). Northern blotting confirmed the absence of PAI-1 mRNA in v-*ras*-transformants and the restoration of mRNA expression in response to NaB (b). 2-D electrophoretic mapping of the SAP fraction proteins derived from ^{35}S-methionine-labeled cultures revealed, furthermore, that PAI-1 induction in response to NaB treatment was rather selective (c). Map positions of the glycosylated PAI-1 isoforms are indicated (solid red outlined rectangle). Proteins common between the cell types are highlighted in color (purple circles, red dashed line box, blue ovals indicating vimentin, and phosho-vimentin breakdown products and actin by black arrows) and did not change in abundance despite the significant PAI-1 induction evident in the v-*ras*/NaB protein profile (c).

cells in a concentration-dependent manner with an 80% reduction in final population density after a 5-day exposure to 100 nM PAI-1 (Figure 5(a)). Indeed, the level of growth inhibition in cultures exposed to 20 nM PAI-1 (45% reduction in population density relative to the corresponding control) (Figure 5(a)) approximated the 47.5% decrease induced by 10 mM NaB even in the presence of serum (Figure 4(b)). Ha-*ras*-transformed HaCaT cells, which express low levels of PAI-1 in response to EGF [33], were also growth inhibited by exposure to PAI-1 14-1B in the presence of FBS or EGF (Figures 5(b) and 5(c)) largely due to G_1 arrest (Figure 5(c)). To assess this effect more critically in a genetic context, antisense knockdown (PAI-1^{KD}; 4HH) cells (Figure 6(a)), which are resistant to NaB-dependent proliferative inhibition, were incubated in PAI-1-supplemented medium with or without, addition of NaB. Recombinant PAI-1, at a final concentration of 20 nM, effectively suppressed PAI-1^{KD} cell proliferation; the combination of PAI-1 + NaB did not significantly impact the extent of cytostasis compared to PAI-1 alone (Figure 6(b)). Transient vector-driven re-expression of PAI-1 in Rc/CMVPAI v-*ras* transfectants (Figure 6(a)) similarly reduced cell growth relative to cells transfected with the empty Rc/CMV construct (Figure 6(b)). Mass cultures of Rc/CMVPAI-expressing cells and, in particular, their G418-selected clonal isolates, but not cells transfected with Rc/CMV without the 2.6 kb PAI-1 cDNA insert, had significant numbers of very well-spread cells (a hallmark of the growth arrest phenotype in renal epithelial cells [14–16]) compared to Rc/CMV populations. The marked reduction in cell proliferation (Figure 6(b)) and increased spreading in Rc/CMVPAI as compared to Rc/CMV transfectants correlated with an approximately 22-fold increase in PAI-1 expression.

4. Discussion

Data mining of microarray and serial analysis of gene expression profiles consistently identified increased PAI-1 levels as characteristic of specific growth arrest states (e.g., [23, 27, 35, 36, 39, 42–46]). Similar to other HDACi-regulated genes, several of which negatively regulate cell cycle progression [10–13], PAI-1 is a particularly relevant candidate as this SERPIN attenuates uPA-/plasmin-dependent growth factor activation and cellular proliferative responses [22, 23], mediates p53-dependent cytostasis [23–26], and is required for TGF-β1-mediated antiproliferative responses [27]. In cells expressing activated *ras* or *raf* oncogenes, moreover, induced PAI-1 initiates the engagement of a senescence-like phenotype [28–31] while, for those cells that escape *ras*-induced senescence, the growth arrest program can be "rescued" upon exposure to certain HDACi (e.g., NaB) with concomitant high-level PAI-1 induction [16, 17, 32]. While molecular events underlying NaB-stimulated PAI-1 expression is unclear, NaB enhances Smad3 phosphorylation and potentiates TGF-β-induced PAI-1 expression [47], concomitant with NaB-induced G_1 arrest [48]. Indeed, overexpression of SMAD3 in v-Ha-*ras*-transformed keratinocytes induced a cytostatic response, stimulated PAI-1 promoter

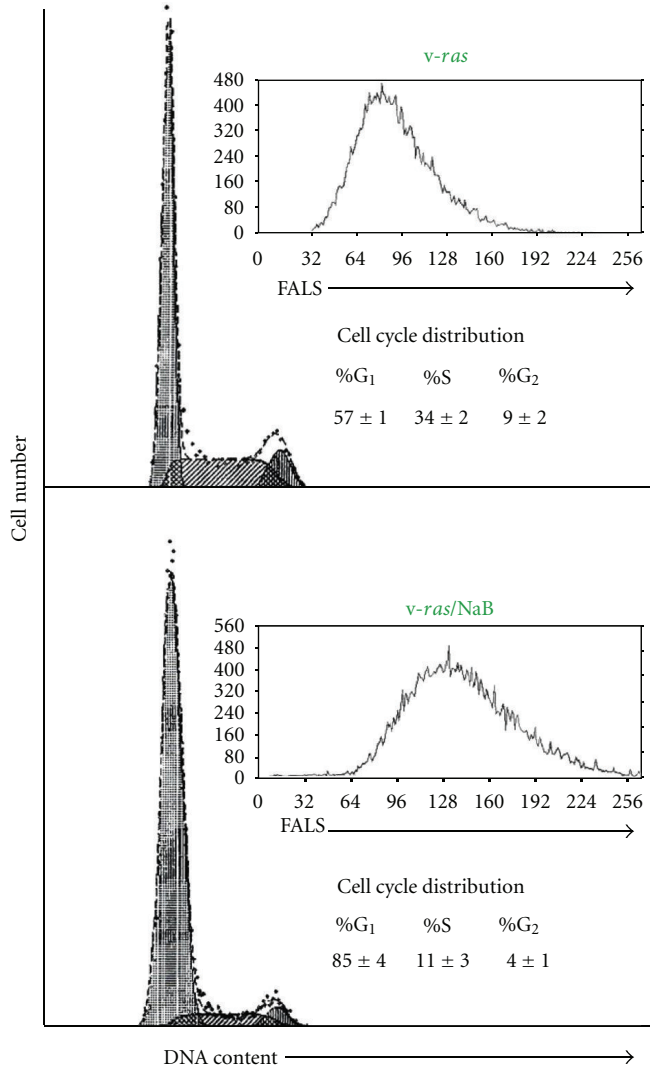

Figure 2: Impact of NaB on cell cycle progression in v-*ras* transformants. The S-phase fraction of exponentially growing (10% FBS) cells approximated >34% with a tight mean cellular size distribution (mean: channel 80) as assessed by forward angle light scatter (FALS) measurements. NaB treatment resulted in a G_1 block (even in FBS-supplemented medium) and a significantly increased mean size.

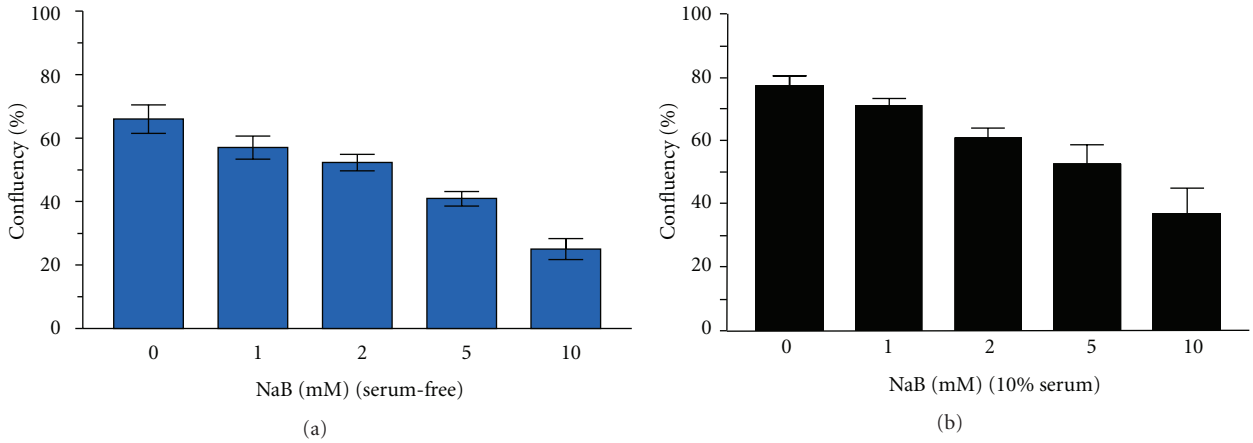

Figure 3: NaB suppressed cell growth in serum-free or supplemented culture conditions. Proliferative restriction was maximal at 10 mM resulting in final population densities of just 47% (a) and 52% (b) compared to respective controls. Data plotted is the mean ± standard deviation for triplicate assessments of final cell densities (i.e., % confluency) for each NaB concentration under the two growth conditions.

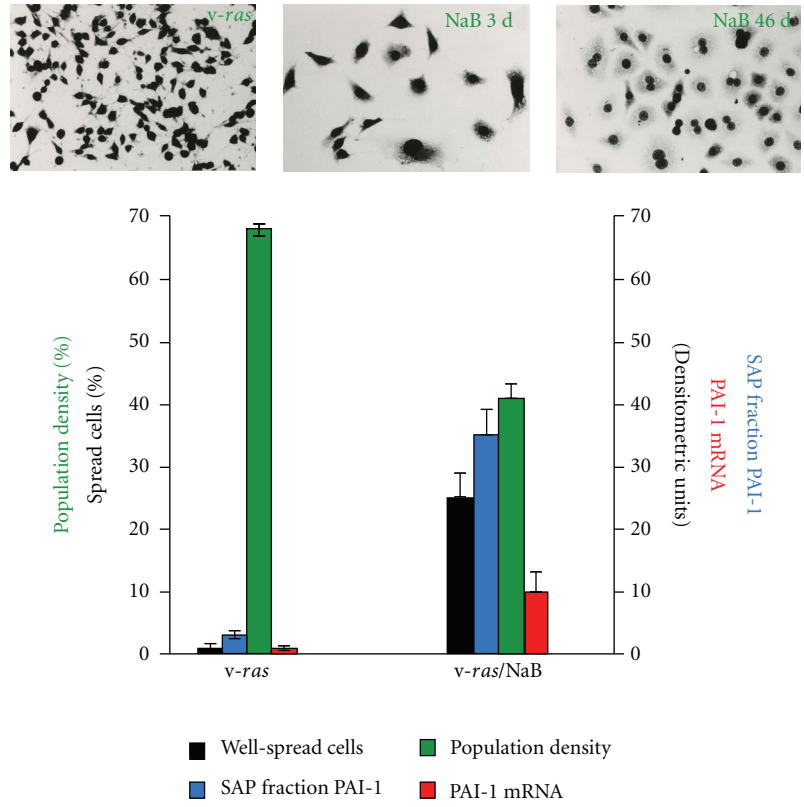

Figure 4: Summary characteristics of the NaB-induced phenotype in v-*ras*-transformed renal epithelial cells. NaB initiated a rapid cytostasis evident within 3 days after exposure with the acquisition of increased cell-spread area consistent with FALS assessment of cell size (i.e., Figure 2). Cells remained growth arrested even after protracted treatment (e.g., 46 days) although such long-term cultures had an increased binucleate frequency. PAI-1 mRNA/protein expression in control populations was low to undetectable in contrast to the levels of PAI-1 transcripts and protein evident in response to NaB. Plots illustrate the mean ± standard deviation for triplicate assessments for each of the parameters evaluated.

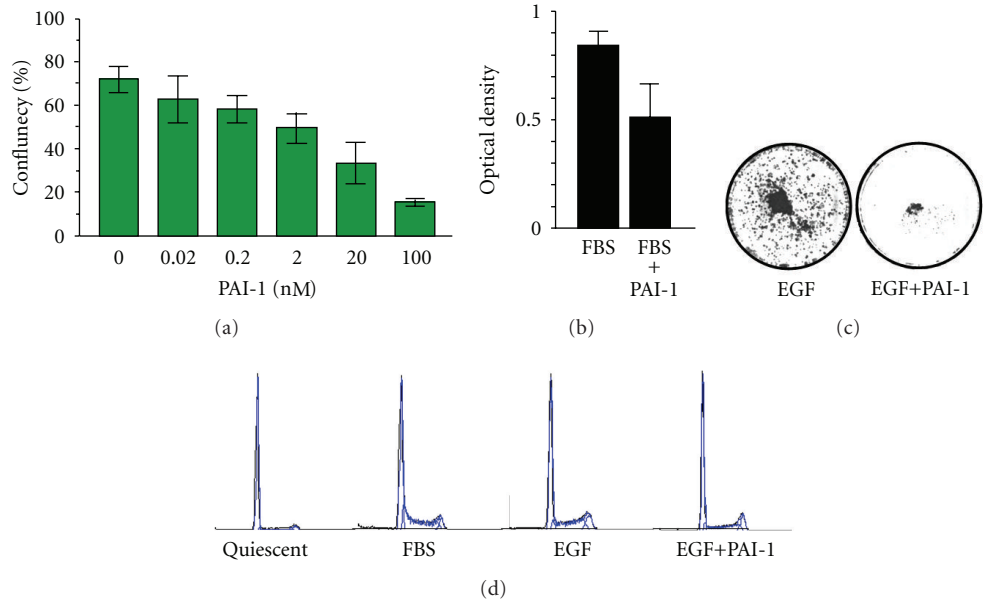

Figure 5: Increasing concentrations of PAI-1 (range 0.02 to 100 nM) effectively suppressed growth of v-*ras*-transformants in serum-free (a) as well as serum-supplemented (b) media. In (b), PAI-1 was added to a final level of 20 nM. PAI-1 also inhibited EGF-induced HaCaT proliferation, as assessed by crystal violet staining of cells stimulated with EGF (10 ng/mL) for 5 days in the absence or presence of PAI-1 (20 nM) (c). Growth arrest in EGF-treated HaCaT keratinocytes reflected a PAI-1-induced G_1 block evident as early as 24 hours after the addition of PAI-1 compared to the prominent S-phase cohort in FBS- or control EGF-treated cultures (d). Starting quiescent populations (1 day in serum-free medium) were virtually devoid of DNA-synthesizing cells.

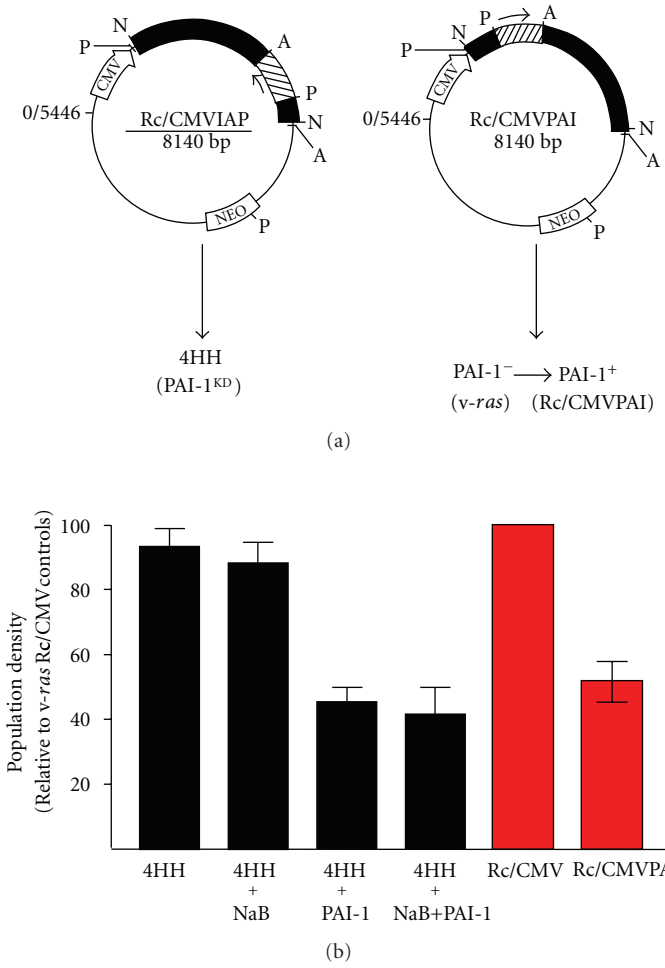

Figure 6: PAI-1 antisense and sense expression vectors were used to generate the PAI-1 knockdown (4HH; PAI-1^{KD}) and overexpressing (Rc/CMVPAI) cell lines, respectively, (a). PAI-1-deficient 4HH cells were resistant to NaB-mediated cytostasis but remained sensitive to PAI-1-induced growth arrest. The combination of NaB+PAI-1 reduced final population densities similar to that of PAI-1 alone cultures (b). Vector-driven PAI-1 overexpression in v-*ras* transformants also inhibited cell growth consistent with results of the PAI-1 add-back experiments (e.g., Figure 5). Data plotted represents the mean ± standard deviation for triplicate assessments of final cell densities (i.e., % confluency).

(3TP-Lux reporter)-dependent transcription, and increased the incidence of senescent epithelial cells [49]. The present findings are consistent with these and previous data that TGF-β-initiated growth inhibition as well as senescence arrest is PAI-1-dependent [23, 27] and establish, moreover, PAI-1 as a mediator of NaB-initiated cytostasis. Whether this response can be adapted for directed "senescence therapy" of human cancers, remains to be assessed.

NaB upregulates the cell cycle inhibitors $p21^{WAF1/CIP1}$ and $p16^{INK4A}$ in human fibroblasts although targeted disruption of p21 only weakly impacted HDACi-induced senescence-like growth arrest. $p53^{-/-}$ mouse embryo fibroblasts (MEFs), moreover, are resistant to NaB-initiated cytostasis indicating that this tumor suppressor is a major senescence determinant in MEFs [50], and NaB-mediated apoptosis in human melanoma cells is p53-dependent [51]. Indeed, nutlin-3, an MDM2 inhibitor which restores p53 function in tumor cells that retain a wild-type p53, cooperate with several HDACis (including NaB) to induce cell death in p53 wild-type tumor cell lines but not in p53-null PC-3 prostatic carcinoma likely by HDACi-induced p53 hyperacetylation and/or MDM2/MDM4 downregulation [52]. This may be dependent, in part, on the extent of increased p53 expression in response to NaB [53]. Similarly, NaB-stimulated p53 transcriptional activity initiated irreversible G_1/S cell cycle arrest in c-Ha-*ras*-transformed rat embryo fibroblasts that were p53 wild-type but not in cells with an inactivated p53 [54]. While the actual contribution of p21 versus INK4A/ARF-encoded genes (e.g., p19) in NaB-induced growth arrest is uncertain [55, 56], the role of p53 (at least in MEFs) may be more relevant since p53 is required for PAI-1 expression and growth arrest (see [27, 57]; and Overstreet et al., in preparation). p53 status, therefore, may be a major aspect of HDACi-induced cell cycle arrest through its transcriptional control of PAI-1 and, thereby, PAI-1-dependent cytostasis.

Acknowledgment

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