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Selective transcription and cellular proliferation induced by PDGF require histone deacetylase activity

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Abstract

Histone deacetylases (HDACs) are key regulatory enzymes involved in the control of gene expression and their inhibition by specific drugs has been widely correlated to cell cycle arrest, terminal differentiation, and apoptosis. Here, we investigated whether HDAC activity was required for PDGF-dependent signal transduction and cellular proliferation. Exposure of PDGF-stimulated NIH3T3 fibroblasts to the HDAC inhibitor trichostatin A (TSA) potently repressed the expression of a group of genes correlated to PDGF-dependent cellular growth and pro-survival activity. Moreover, we show that TSA interfered with STAT3-dependent transcriptional activity induced by PDGF. Still, neither phosphorylation nor nuclear translocation and DNA-binding in vitro and in vivo of STAT3 were affected by using TSA to interfere with PDGF stimulation. Finally, TSA treatment resulted in the suppression of PDGF-dependent cellular proliferation without affecting cellular survival of NIH3T3 cells. Our data indicate that inhibition of HDAC activity antagonizes the mitogenic effect of PDGF, suggesting that these drugs may specifically act on the expression of STAT-dependent, PDGF-responsive genes.

Keywords: Tyrosine kinase receptors; Phosphorylation; Histone deacetylase inhibitors; STAT activation; Oncogenes

Platelet-derived growth factor (PDGF) is a major mitogen for fibroblasts, smooth muscle cells, and other cell types [1]. Thus, signaling initiated by its cognate receptor has been widely used as a very powerful model system for the study of the signal transduction mechanisms controlling cell cycle progression induced by tyrosine kinase receptors (RTKs) [1] and for the understanding of the molecular basis of cellular proliferation. Not surprisingly, since when twenty years ago PDGF was identified as the cellular homologue of the transforming retroviral v-*sis* oncogene [2], genetic alterations have been characterized which cause constitutive activation of PDGF receptors, autocrine growth stimulation and consequently, human cancer [3]. Consequently, signaling pathways stimulated by this growth factor have been always considered interesting targets for cancer treatment.

In eukaryotic cells, histone proteins organize DNA into nucleosomes, which are regular repeating structures of chromatin [4]. In general, DNA-histone interactions condense chromatin and repress transcription, while reduction of these interactions relaxes chromatin and enhances gene transcription, by increasing the access to the DNA of proteins such as RNA polymerase and transcription factors [5]. Specifically, histone acetylation neutralizes the positive charge of conserved lysine residues within the NH_2-terminal domains of the core histones, therefore diminishing interactions between the negatively charged DNA and the histones [6]. Two classes of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), reversibly regulate the extent of such modifications [4]. Different studies have recently demonstrated that histones are not the only proteins under the control of HATs and HDACs [4,7]. Thus, substrates for acetylation now include several

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transcription factors, cytosolic proteins such as Tubulin, and proteins that shuttle from the nucleus to the cytoplasm such as Importin [7,8]. The control by acetylation of the activity and stability of these substrates and, in particular, of transcription factors, therefore suggests that HATs and HDACs are able to control gene expression also by mechanisms that are distinct from their direct effect on chromatin.

HDAC inhibitors were initially discovered for their ability to reverse the malignant phenotype of transformed cells in culture [5]. Since then, huge efforts have been made to unravel the identity of the genes controlled by such compounds. Several structural classes of HDAC inhibitors have been identified, including short-chain fatty acids such as valproic acid (VPA) and sodium butyrate, cyclic tetrapeptides such as trapoxin A and benzamides, and hydroxamic acids such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) [4]. As expected from their ability to stimulate gene expression by acting on histones, HDAC inhibitors induce the levels of different tumor suppressor genes (i.e., p53, p21, and gelsoline) that cause cell cycle arrest in G_1 and/or G_2, apoptosis and/or differentiation [6]. Still, more recently it has become clear that they are also able to inhibit the expression of tumor activators such as VEGF [5], c-Myc, Bcl-X_L, and HIF-1 [9], suggesting for these drugs a mechanism of action more complex than the mere effect on histone acetylation.

Materials and methods

Reagents. Human recombinant PDGF (Intergen) was used at a final concentration of 12.5 ng ml^{-1}. The HDAC inhibitors sodium butyrate (Sigma), SAHA and TSA (Biomol) were added to the cells 30 min before stimulation at the indicated concentrations. Staurosporine (Sigma) was used at a final concentration of 0.5 μM. The STAT-responsive element (×4) luciferase vector (pStat-Luc) was kindly provided by J.E. Jr. Darnell [10]. PCR amplification of the wild-type STAT3 was cloned in the pCEFL-AU1 expression vector. Specific maps and restriction sites will be made available upon request.

NIH3T3 fibroblasts were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) bovine calf serum (BioWhittaker), 2 mM L-glutamine, and penicillin-streptomycin (Invitrogen). RAT2 fibroblasts (kindly provided by G. De Vita) were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Invitrogen), 2 mM L-glutamine, and penicillin-streptomycin (Invitrogen).

Northern blot analysis. After 24 h of starvation, NIH3T3 cells were stimulated with 12.5 ng ml^{-1} PDGF for various times in absence or after pre-treatment with increasing concentrations of TSA. Samples were then processed as previously described [11]. As c-*myc* probe, we used a 450-bp PstI DNA fragment from the human c-*myc* gene (pcDNAIII/GS-Myc-V5, purchased from Invitrogen). As *VEGF* probe, we used a 500-bp *Bam*HI fragment from the human *VEGF* cDNA (pCEFL-P-VEGF). As *bcl-X_L* probe, we used a 500-bp *Eco*RI fragment from the *bcl-X_L* cDNA (pcDNA4/TO-Bcl-X_L, kindly provided by I. Iaccarino). As c-*jun* probe, we used the complete 1000-bp *Eco*RI-Not I c-jun cDNA (pCEFL-AU1-c-Jun). The RNA membranes were pre-hybridized for 2 h in hybridization solution (ExpressHyb, Clontech) at 70 °C. The ^{32}P-labeled probe for the human c-*myc* gene was added to the blots and hybridized for another 16 h at 60 °C. The ^{32}P-labeled probes of the c-*jun*, *VEGF*, and *bcl-X_L* genes were added to the blots and hybridized for another 16 h at 68 °C. The blots were washed in accordance with the manufacturer's specifications (ExpressHyb, Clontech). Accuracy of RNA loading and transfer was confirmed by fluorescence under ultraviolet light after staining with ethidium bromide.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, NY), in accordance with the manufacturer's instructions. Briefly, chromatin from NIH3T3 cells was fixed by directly adding formaldehyde (1% final) to the cell culture media. Nuclear extracts were isolated from the cells and then sonicated to obtain mechanical sharing of the fixed chromatin. Transcription factor-bound chromatin was immunoprecipitated with specific antibodies, cross-linking was reversed, and the isolated genomic DNA was amplified by PCR, using specific primers encompassing the murine c-*myc* promoter: forward AP66 (5′-ATACCTGTGACTATTCATTT-3′); reverse AP67 (5′-GATGCTTC CTTGCCTAAGAC-3′). The PCR products were separated on a 2% agarose gel.

Preparation of nuclear and cytoplasmic extracts. Nuclear and cytoplasmic extract preparation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (PIERCE Biotechnology), in accordance with the manufacturer's instructions.

5-Bromo-2′deoxy-uridine assay. NIH3T3 cells were starved for 24 h, stimulated with 12.5 ng ml^{-1} PDGF for 15 h in absence or after 30 min pre-treatment with increasing concentrations of TSA, before incubation with 5-Bromo-2′deoxy-uridine (BrdU) (10 mmol) for 4 h. The BrdU assays were performed using the 5-Bromo-2′Deoxy-uridine Labeling and Detection Kit I (ROCHE), in accordance with the manufacturer's instructions. The slides were mounted in Gel-mount (Biomeda Corp.) and examined with a Zeiss Axiophot photomicroscope equipped with epifluorescence.

Electrophoretic mobility shift assays. Nuclear extracts were obtained from NIH3T3 cells, starved overnight, and then stimulated with PDGF for various times in absence or after pre-treatment with increasing concentrations of TSA. Samples were then processed as previously described [12]. Complementary synthetic oligonucleotides containing the STAT3 consensus sequence from Santa Cruz Biotechnology were labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase (USB). Labeled oligonucleotides were purified using G25 columns (Amersham Biosciences) and used as probes. Complexes were analyzed on non-denaturing (5%) polyacrylamide gels in TBE buffer (40 mM Tris, 270 mM glycine, and 2 mM EDTA, pH 8.0) and run at 13 V/cm at 4 °C. For supershift assays, 1 μg of the indicated antiserum was added to the binding reaction.

Western blot analysis and antibodies. Lysates of total cellular proteins were analyzed by protein immunoblotting after SDS–PAGE with specific rabbit antisera or mouse monoclonal antibodies. Immunocomplexes were detected by the ECL Plus Reagent (Amersham Biosciences), by using goat antiserum against rabbit or mouse IgG coupled to horseradish peroxidase (Amersham Biosciences). Electrophoretic mobility shift assays (EMSA) and Western blot analysis were performed using rabbit polyclonal antibodies against STAT3-[pSer727] (BIOSOURCE), STAT3-[pTyr705] (Cell Signaling Technology), H3 (Novous Biologicals), STAT3, Acetyl-Histone H4, and Acetyl-Histone H3 (UPSTATE), Rac1, Cdk2, Histone H4, and STAT3 C20-X (Santa Cruz Biotechnology).

Reporter gene assays. For each well, cells were transfected with different expression plasmids together with 200 ng of the indicated reporter plasmid and 10 ng pRL-null as an internal control. In all of the cases, the total amount of plasmid DNA was adjusted with empty vector. After 16–20 h from transfection, firefly and *Renilla* luciferase activities present in cellular lysates from serum-starved cells were assayed using the Dual-luciferase reporter system (Promega) and light emission was quantified using the 20/20^n luminometer (Turner BioSystems). Data obtained by firefly luciferase, which correspond to absolute activities of the reporter plasmid, were normalized for the corresponding transfection efficiency by using the *Renilla* luciferase activity of each sample.

Immunofluorescences. NIH3T3 cells were starved for 24 h, stimulated with 12.5 ng ml^{-1} PDGF for 1 h in absence or after pre-treatment for 30 min with increasing concentrations of TSA. The cells were fixed for 10 min in 2% paraformaldehyde-1% sucrose solution at room temperature. Incubation with anti-STAT3 antibodies (Upstate Biotechnology) was

performed in accordance with the manufacturer's instructions. Slides were washed with PBS and incubated with a secondary anti-rabbit antibody conjugated to FITC (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. The slides were mounted in Gel-mount (Biomeda Corp.) and examined with a Zeiss Axiophot photomicroscope equipped with epifluorescence.

Results

HDAC inhibitors impede the PDGF-dependent expression of different growth-promoting genes

A huge amount of data clearly demonstrate that HDACs are able to modulate, both up- and down-regulating, the expression of a vast number of genes [9,13,14]. Indeed, differently from what was expected from their role on chromatin condensation, suppression of HDAC activity by different classes of specific inhibitors has clearly demonstrated that these enzymes can also function as activators of gene transcription [15]. As an approach to examine the role of acetylation in PDGF-dependent transcriptional activity, in NIH3T3 cells, we took advantage of the availability of pharmacological inhibitors of HDACs [4]. In particular, trichostatin A (TSA) potently and specifically inhibits HDACs causing an accumulation of acetylated histone species in a variety of mammalian cell lines [16].

We first sought to confirm the ability of this compound to affect histone acetylation in our cellular system. Indeed, as shown in Fig. 1, TSA strongly induced, in a dose-dependent manner, histone H3 and histone H4 acetylation, after 18 h treatment.

To examine the role of HDACs in PDGF-dependent transcription, we therefore assessed the ability of PDGF to modulate the expression of a group of genes correlated to cell growth, angiogenesis, and cell survival, namely *c-myc*, *VEGF*, *bcl-X_L*, and c-*jun*. Cells were starved for 24 h and then stimulated with PDGF for up to 7 h. Northern blot analysis of the extracted total RNA showed an increase in the levels of the four genes, although at different time-points after PDGF stimulation. Indeed, while PDGF caused a peak of c-*myc* and c-*jun* mRNAs after one hour of treatment (Figs. 2A and D), such increase was delayed for *VEGF* (3 h) (Fig. 2B) and *bcl-X_L* (7 h) (Fig. 2C). Next, we analyzed the effect of TSA on the expression of the different genes. While the increase of the expression of c-*myc*, *VEGF*, and *bcl-X_L* was strongly inhibited by 30 min pretreatment with increasing concentrations of TSA (Figs. 2E–G), c-*jun* expression was unaffected by such treatment (Fig. 2H) establishing a role for acetylation in specific early and late gene expression processes controlled by PDGF.

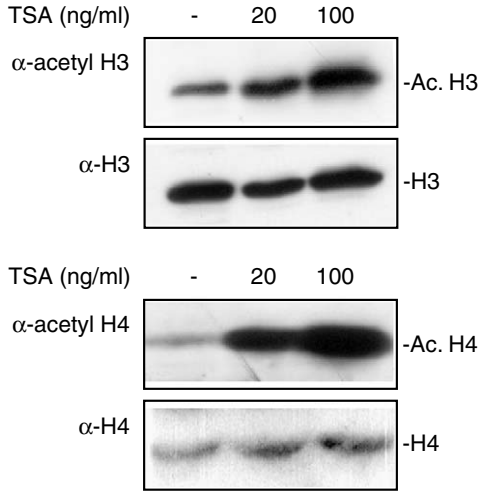

Fig. 1. TSA induces histone H3 and H4 acetylation. NIH3T3 cells were starved for 24 h and pretreated or not with increasing concentrations of TSA for 15 h. Nuclear extracts were then assayed by Western blot, using the specific anti-acetyl-Histone H3 (upper panel) and anti-acetyl-Histone H4 (bottom panel) antibodies. -, No treatment.

To confirm that inhibition of gene expression was the result of the HDAC inhibitory activity of TSA, other two drugs, SAHA and sodium butyrate, were used in similar experiments, to assess their ability to interfere with PDGF-dependent stimulation of c-*myc* expression. In particular, it is important to notice that, while TSA and SAHA belong to the same chemical class of HDAC inhibitors, sodium butyrate is a chemically different compound [4]. As shown in Figs. 3A and B, both SAHA and sodium butyrate were very efficient in blocking PDGF-induced c-*myc* expression, therefore strongly supporting that the effects observed for TSA on gene expression represent a general feature and are dependent on its histone deacetylase inhibitory activity. As additional controls, the effects in time-courses of TSA and sodium butyrate were also investigated on the expression of the c-*myc* proto-oncogene, used as a model for the response of PDGF-regulated genes to HDAC inhibitory drugs. As shown in Figs. 3C and D, treatments of NIH3T3 cells up to 60 min with both TSA and sodium butyrate did not affect c-*myc* expression. Ultimately, to ascertain a role for HDAC activity in cellular models different from NIH3T3 cells, we treated with TSA PDGF-stimulated RAT2 fibroblasts, demonstrating that also in this cell line the HDAC signaling network is able to control PDGF-dependent gene expression (Fig. 3E). Altogether, these results show that deacetylase activity is important for both the early and late specific PDGF-dependent transcriptional programs.

TSA interferes with STAT-dependent transcriptional activity induced by PDGF

Signal transducers and activators of transcription (STATs) constitute an evolutionarily conserved family of transcription factors, originally identified as mediators of cytokine signaling [17]. Still, STAT proteins are also activated by polypeptide growth factors such as PDGF and epidermal growth factor (EGF) [18,19]. STAT proteins are involved in the regulated expression of numerous genes underlying diverse cellular processes ranging from immune response to antiviral protection, apoptosis, proliferation, differentiation, and cell survival [17]. Among the regulated genes, expression of growth-controlling genes

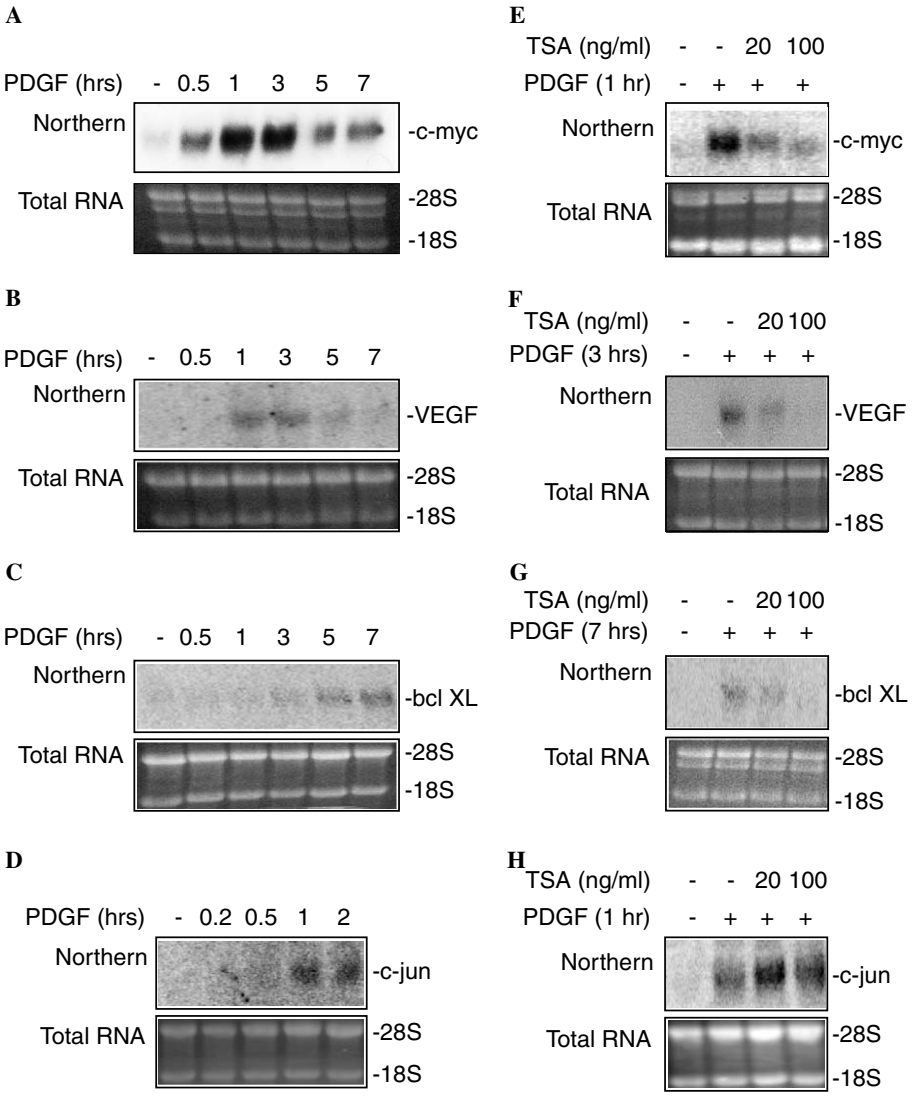

Fig. 2. HDAC inhibitors prevent PDGF-dependent expression of different growth-promoting genes. Analysis of c-*myc* (A), *VEGF* (B), *bcl-X*$_L$ (C), and c-*jun* (D) mRNA expression in NIH3T3 cells stimulated for the indicated durations with PDGF. Analysis of c-*myc* (E), *VEGF* (F), *bcl-X*$_L$ (G), and c-*jun* (H) mRNAs in NIH3T3 cells pretreated with increasing concentrations of the specific HDAC inhibitor, TSA, and then stimulated for 1 h with PDGF. -, No treatment.

such as *cyclin D1*, *bcl-X*$_L$, *VEGF*, and c-*myc* has been correlated to STAT activity [20,21]. Aberrant STAT signaling may also participate in development and progression of human cancers [22]. Indeed, several studies have shown that abrogation of STAT3 activity or expression by use of dominant negative inhibitors or antisense oligonucleotides leads to reversal of the malignant phenotype and apoptosis [22].

In search for a mechanism that could explain the ability of TSA to inhibit PDGF-dependent gene expression, we noticed that all investigated genes inhibited by TSA, c-*myc*, *VEGF*, and *bcl-X*$_L$ are under the control of STAT transcription factors [23–25]. As numerous recent reports point to a positive role for HDACs in cytokine- and STAT-dependent gene regulation [6], we decided to investigate the possibility that inhibition of HDACs by TSA blocked gene expression by directly inhibiting STAT activity. To test this hypothesis, we took advantage of the availability of a STAT-dependent luciferase reporter construct, pStat-Luc [10]. NIH3T3 cells were therefore transiently transfected with this reporter, left untreated or treated with increasing concentrations of TSA and PDGF (6 h), alone or in combination (Fig. 4A). While PDGF strongly induced STAT activity in these cells, TSA almost abolished such response (Fig. 4A), therefore suggesting that HDAC activity is required for optimal PDGF-dependent STAT activation.

To establish a requirement for HDAC activity for the transcriptional function of a specific STAT family member, we also performed a similar experiment in presence of transiently transfected STAT3. Due to the very high levels of this protein, the observed STAT transcriptional activity in the transfected cells could be referred to the overexpressed protein with little or no influence of different

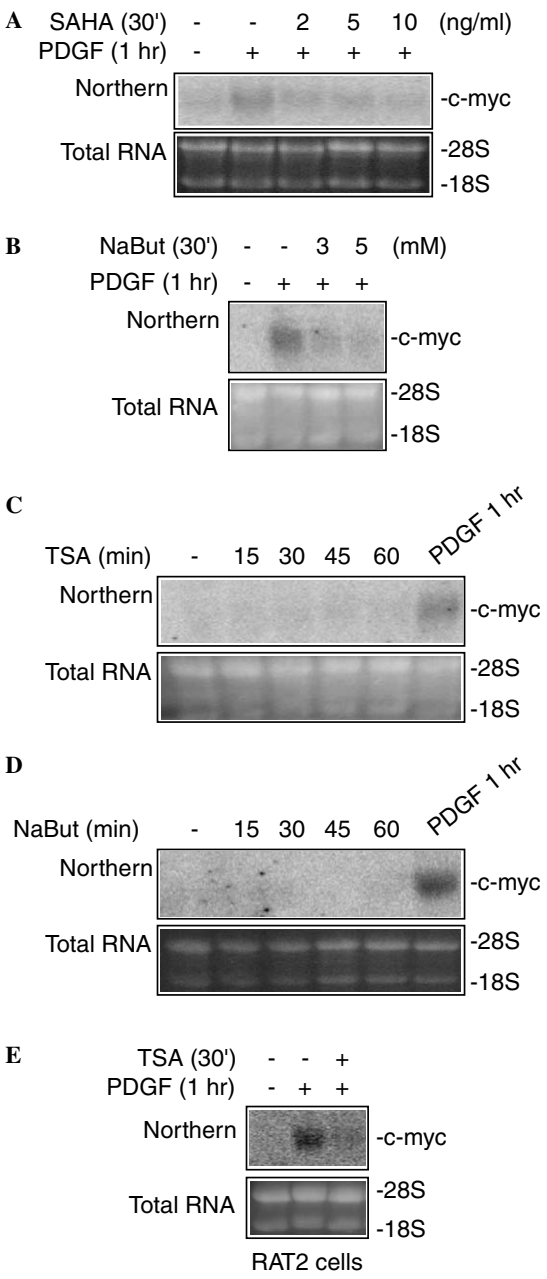

Fig. 3. (A,B) Analysis of c-*myc* mRNA in NIH3T3 cells pretreated with increasing concentrations of the specific HDAC inhibitors, SAHA (A) and sodium butyrate (NaBut) (B), and then stimulated for 1 h with PDGF. (C,D) Analysis of c-*myc* mRNA expression in NIH3T3 cells stimulated for the indicated durations with TSA and sodium butyrate (NaBut). (E) Analysis of c-*myc* mRNA in RAT2 cells pretreated with TSA (100 ng/ml). -, No treatment.

endogenous STAT family members. We therefore cotransfected NIH3T3 cells with pStat-Luc and an expression vector for STAT3, and then left untreated or treated with increasing concentrations of TSA and PDGF (6 h), alone or in combination (Fig. 4B). Again, while PDGF strongly induced STAT3 activity in these cells, TSA almost abolished such response (Fig. 4B), therefore suggesting that HDAC activity is required for optimal PDGF-dependent activation of STAT3.

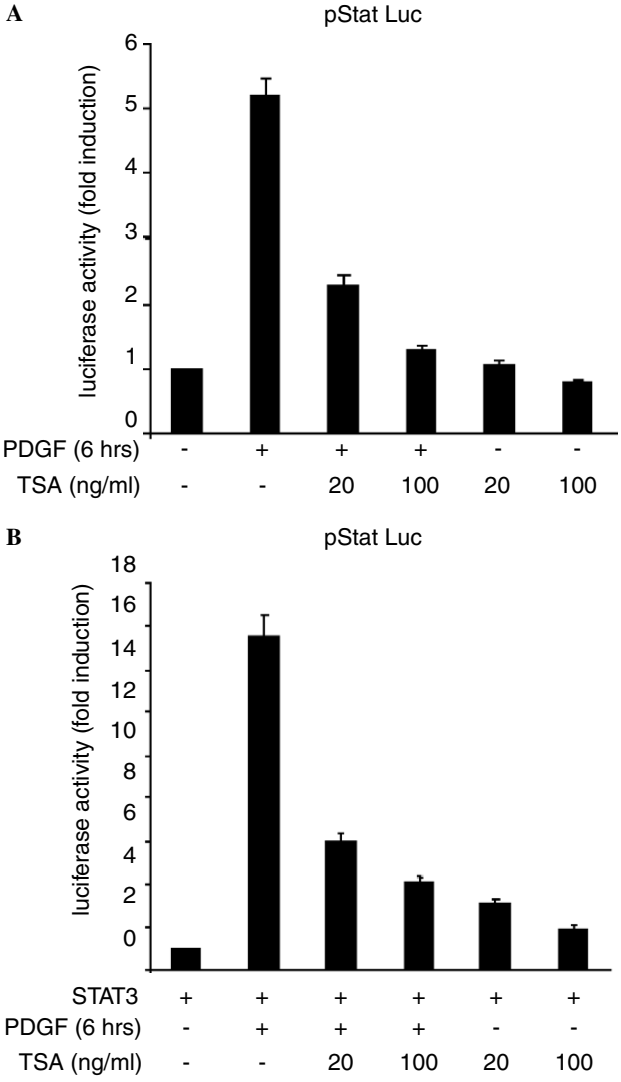

Fig. 4. TSA inhibits STAT transcriptional activity induced by PDGF. (A) NIH3T3 cells were transfected by the lipofectAMINE reagent (Invitrogen) with a STAT-responsive luciferase construct, pStat-Luc (200 ng). The day after transfection, cells were left untreated or treated with combinations of increasing concentrations of TSA and PDGF (6 h), as indicated. (B) Same as in (A), but cotransfecting cells with pStat-Luc and an expression vector for STAT3.

Inhibition of HDAC activity does not directly affect STAT3 phosphorylation, nuclear translocation, and DNA-binding

Activation of STAT proteins necessarily requires phosphorylation of cytosolic STAT monomers on a single tyrosine residue at their C-terminus [17]. Once phosphorylated, STAT proteins dissociate from the receptors and form homo- or heterodimers that translocate to the nucleus where they interact with other transcriptional modulators bound to specific promoter sequences [17]. Some STATs also require phosphorylation on a conserved serine residue for maximal transcriptional activation [26].

To determine whether HDAC inhibition alters PDGF-stimulated tyrosine and/or serine phosphorylation of STAT3, we performed Western blot analysis with

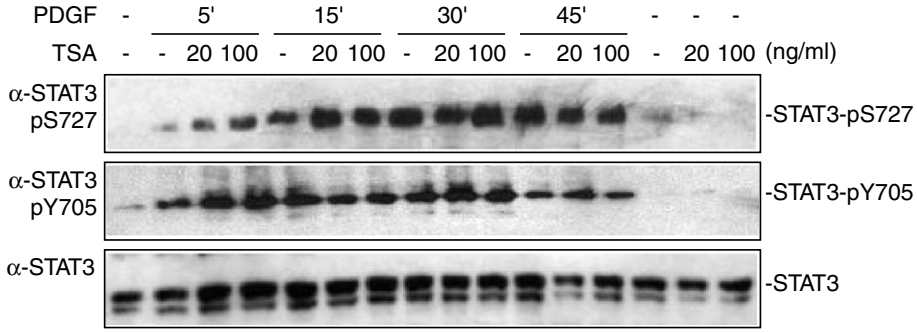

Fig. 5. TSA does not interfere with STAT3 tyrosine[705] and serine[727] phosphorylation upon PDGF stimulation. NIH3T3 cells were starved for 24 h and then left untreated or treated with combinations of increasing concentrations of TSA and PDGF, as indicated. Total lysates were assayed by Western blot using specific antibodies: anti-STAT3-[pSer727] (upper panel), anti-STAT3-[pTyr705] (middle panel), and anti-STAT3 (bottom panel). -, No treatment; α-, antibody against.

phospho-specific antibodies directed against tyrosine[705] or serine[727] of this protein. Cells were incubated with PDGF for up to 45 min, with or without pretreatment for 30 min with increasing concentrations of TSA. As shown in Fig. 5, PDGF-dependent phosphorylation of STAT3 was not affected by TSA, when normalized to endogenous STAT3 protein levels.

To determine weather treatment with TSA affects STAT3 sub-cellular localization, NIH3T3 cells were treated with PDGF alone or in association with TSA and then examined by immunofluorescence analysis using a specific anti-STAT3 antibody. In our system, TSA treatment of PDGF-stimulated cells had no effect on sub-cellular localization of STAT3 (Fig. 6A). To confirm these data, we also prepared cytoplasmic and nuclear extracts from NIH3T3 cells treated with PDGF alone or in association with TSA and then analyzed them for the presence of the STAT3 protein. As shown in Figs. 6B and C, TSA treatment of PDGF-stimulated cells had no effect on sub-cellular localization of STAT3. In this regard, it is important to notice that, although even PDGF could not induce nuclear translocation of STAT3 it has been clearly demonstrated that, in specific cellular systems, STAT proteins are equally distributed between the cytoplasm and the nucleus, and such balance is not affected by stimuli that are able to activate them [27].

Next, we sought to investigate, by electrophoretic mobility shift assay (EMSA), the possibility that inhibition of HDAC activity could directly affect STAT3 DNA-binding ability. As expected, PDGF stimulation rapidly induced the binding of STAT homo- and hetero-dimers to a double-strand oligonucleotide containing a typical STAT-responsive element (Fig. 7A), reaching a peak at 15 min. Specifically, three major STAT-containing DNA-binding complexes were observed, represented by STAT3 homo-dimers (slowest migrating complexes), STAT1 homo-dimers (fastest migrating complexes), and STAT1/STAT3 hetero-dimers (complexes with intermediate mobility) [28] (Fig. 7A). To further verify the presence of STAT3 in such complexes, we also performed supershift analysis by incubating the binding reactions with antibodies specific to the STAT3 protein (Fig. 7B). As an additional control, we also verified that the binding of the complexes to the DNA was specific, as it was efficiently competed by addition of an excess of unlabeled oligonucleotide (Fig. 7C). As shown in Fig. 7D, pretreatment of NIH3T3 cells with progressively increasing concentrations of TSA ultimately indicated that the activity of HDACs had no effect on PDGF-stimulated STAT3 (and STAT1) DNA-binding activity.

On the basis of the binding observed in vitro, we next examined by chromatin immunoprecipitation (ChIP) analysis whether TSA could control the binding, in vivo, of STAT3 to the endogenous c-*myc* promoter. In NIH3T3 cells, ChIP assays confirmed that the activity of HDACs had no effect on PDGF-stimulated STAT3 in vivo DNA-binding activity (Fig. 7E), as already observed in vitro by EMSA. Our data therefore indicate that inhibition of HDAC activity does not directly affect the mechanisms by which PDGF activates STAT3 or stimulates its DNA-binding ability.

Inhibition of HDAC activity prevents PDGF-dependent cellular proliferation

HDAC inhibitors have repeatedly demonstrated their efficacy to arrest cellular growth of multiple cell lines [4,5]. Based on the evidence that these drugs profoundly affected PDGF-dependent expression of different genes related to proliferation and survival (see above), we sought to examine the role of acetylation on PDGF-induced NIH3T3 cell proliferation. To this aim, we analyzed bromodeoxyuridine (BrdU) incorporation in quiescent NIH3T3 cells stimulated with PDGF in absence or after pre-treatment with increasing concentrations of TSA. Cells were first starved for 24 h to arrest them in the G_0/G_1-phase of the cell cycle, left untreated or pre-treated with increasing concentrations of TSA, and then stimulated with PDGF for 15 h, a timeframe in which NIH3T3 cells enter S-phase and duplicate their DNA [29]. As shown in Fig. 8, the addition of TSA was able to completely inhibit S-phase progression of stimulated NIH3T3 cells (middle

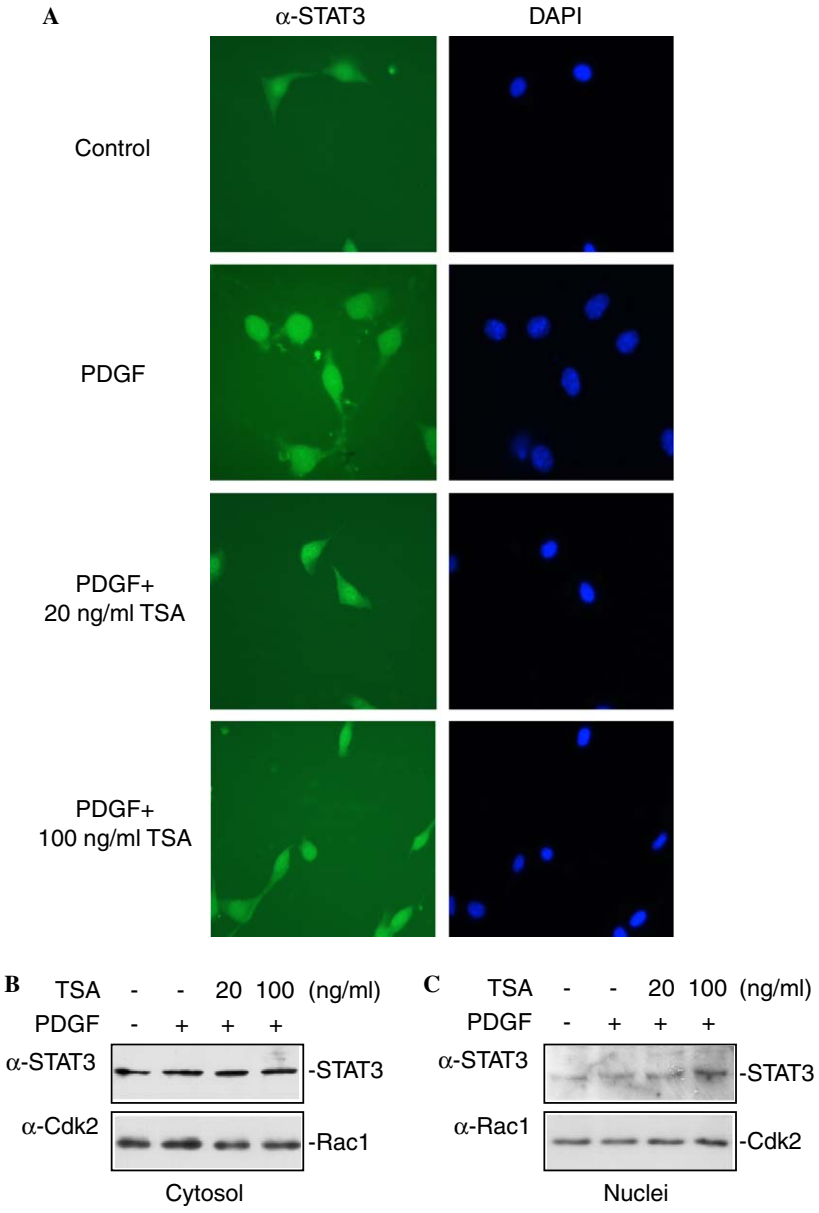

Fig. 6. TSA does not alter the sub-cellular localization of STAT3. (A) NIH3T3 cells were seeded on coverslips and, after 24 h, transferred to serum-free medium for an additional 18 h. Cells were pretreated with increasing concentrations of TSA and then stimulated with PDGF for 1 h. Subsequently, cells were fixed and analyzed by immunofluorescence for endogenous STAT3 (α-STAT3) and nuclear staining with DAPI. (B,C) NIH3T3 cells were transferred to serum-free medium for 18 h. Cells were then pretreated with increasing concentrations of TSA and then stimulated with PDGF for 1 h. Subsequently, both cytoplasmic (B) and nuclear (C) extracts were prepared and cells were fixed and analyzed by Western blot using specific antibodies: anti-STAT3 and anti-Rac1 for cytoplasmic extracts (B); anti-STAT3 and anti-Cdk2 for nuclear extracts (C). -, No treatment; α-, antibody against.

panels). Conversely, as a control, it did not affect cell survival as compared to staurosporine treatment of the cells (Fig. 8, right panels), confirming previous observation that HDAC inhibitors do not induce apoptosis in untransformed cells [30,31] such as NIH3T3. TSA was therefore a strong inhibitor of the early phases of cell-cycle progression of PDGF-stimulated cells.

Discussion

Although several genetic defects exist in human tumors, they frequently seem to converge on limited number of signal transduction pathways often controlling the expression of different cancer-promoting genes. The possibility to modulate the expression of such genes has therefore become a rational target for the treatment of cancer. In recent years, a number of structurally divergent classes of HDAC inhibitors have been identified that induce cell-cycle arrest, terminal differentiation, and/or apoptosis in various cancer cell lines and inhibit tumor growth in animal models [7,8]. Though, in this respect, it is important to note that several non-histone proteins, among which different transcription factors, are direct substrates of acetylation and, in turn, of HDACs [7,8].

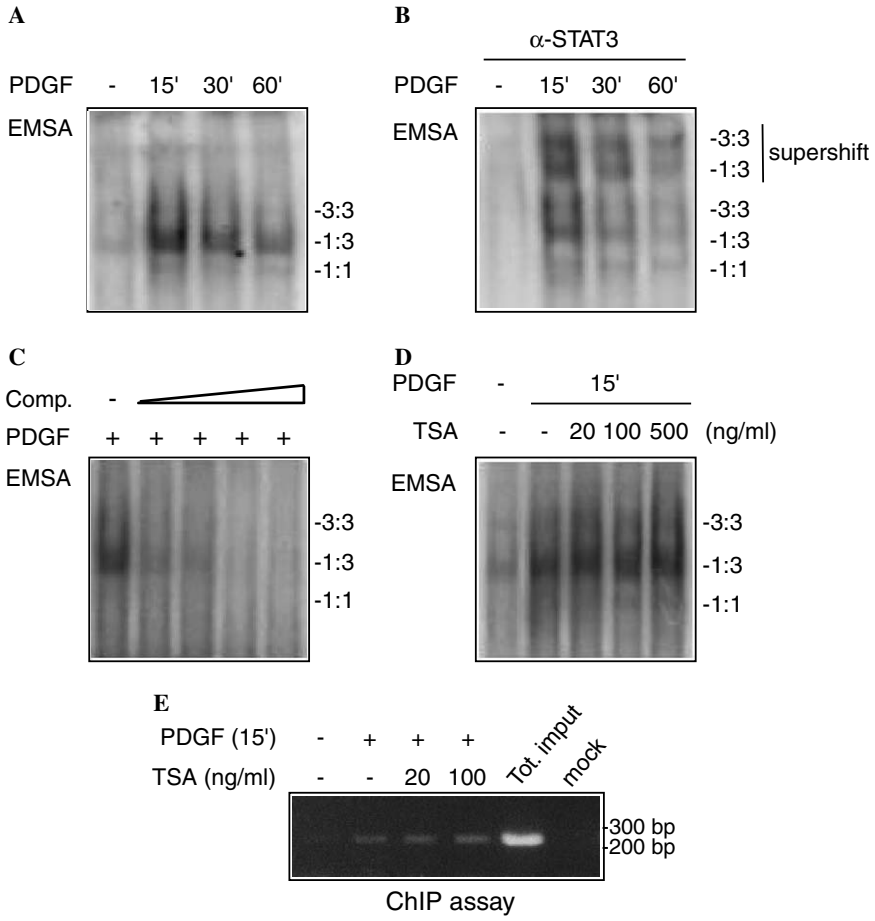

Fig. 7. PDGF-stimulated STAT3 DNA-binding activity is independent of HDAC activity. NIH3T3 cells were serum-starved for 24 h and then treated as indicated. (A) Nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) with a ^{32}P-labeled probe containing a STAT3-responsive element. (B) Supershift analysis with a monoclonal anti-STAT3 antibody of NIH3T3 nuclear extracts, upon PDGF stimulation for the indicated duration. (C) Control of specificity for the EMSA, using the unlabeled oligonucleotides as competitors in concentration 5- to 50-fold-molar excess versus the probe. NIH3T3 cells were stimulated for 15 min with PDGF. (D) EMSA of NIH3T3 nuclear extract upon pretreatment with increasing concentrations of the TSA and stimulation with PDGF for 15 min. The position of the complexes containing STAT3/STAT3 (3:3) and STAT1/STAT1 (1:1) homo-dimers, and STAT1/STAT3 (1:3) hetero-dimers is indicated. (E) ChIP analysis of the murine c-*myc* promoter from NIH3T3 cells pretreated with increasing concentrations of the TSA and stimulated with PDGF for 15 min. Immunoprecipitates from each sample were analyzed by PCR using primers for the murine c-*myc* promoter, as specified in Materials and methods. As a control, a sample representing linear amplification of the total input chromatin was included in the PCR (T.I.). An additional control was performed by amplification of a sample without chromatin (mock). -, No treatment; α-, antibody against; Comp., competitor.

By using TSA, SAHA, and sodium butyrate, we show that deacetylase activity is necessary for the expression of genes correlated to the growth stimulatory (c-*myc*), anti-apoptotic (*bcl-X$_L$*), and pro-angiogenetic (*VEGF*) activity of PDGF. Reasonable candidates for mediating the inhibition of the expression of such genes are STAT family members. Indeed, all the investigated genes inhibited by TSA are under the control of these transcription factors [23–25], while c-*jun*, another PDGF-regulated gene whose promoter is not controlled by STATs, is not sensitive to the HDAC inhibitor treatment. These data therefore suggest that HDAC inhibitors may negatively influence the activity of STAT proteins. Although many papers have recently addressed the role of HDACs in the regulation of STAT activation, the issue is far from being solved. Indeed, many contrasting data are present in the literature relative to the effect of HDAC inhibitors on STAT family members: while in some systems HDAC inhibitors interfere with STAT tyrosine and serine phosphorylation and nuclear translocation [32,33], other papers indicate that these drugs have no direct effect on nuclear translocation, DNA-binding activity, and tyrosine and serine phosphorylation of STAT proteins [15,34,35]. Complicating even more this story, recent data report that, upon cytokine stimulation, STAT3 undergoes acetylation of a single amino acid residue providing an alternative mechanism for its activation [36,37], altogether pointing to a positive role for HDACs in STAT-dependent gene transcription. In our experimental conditions, HDAC activity seems to positively affect STAT activation as TSA strongly inhibits PDGF-dependent activation of STAT transcriptional potential, in particular of STAT3, in NIH3T3 cells. These data therefore support a role for

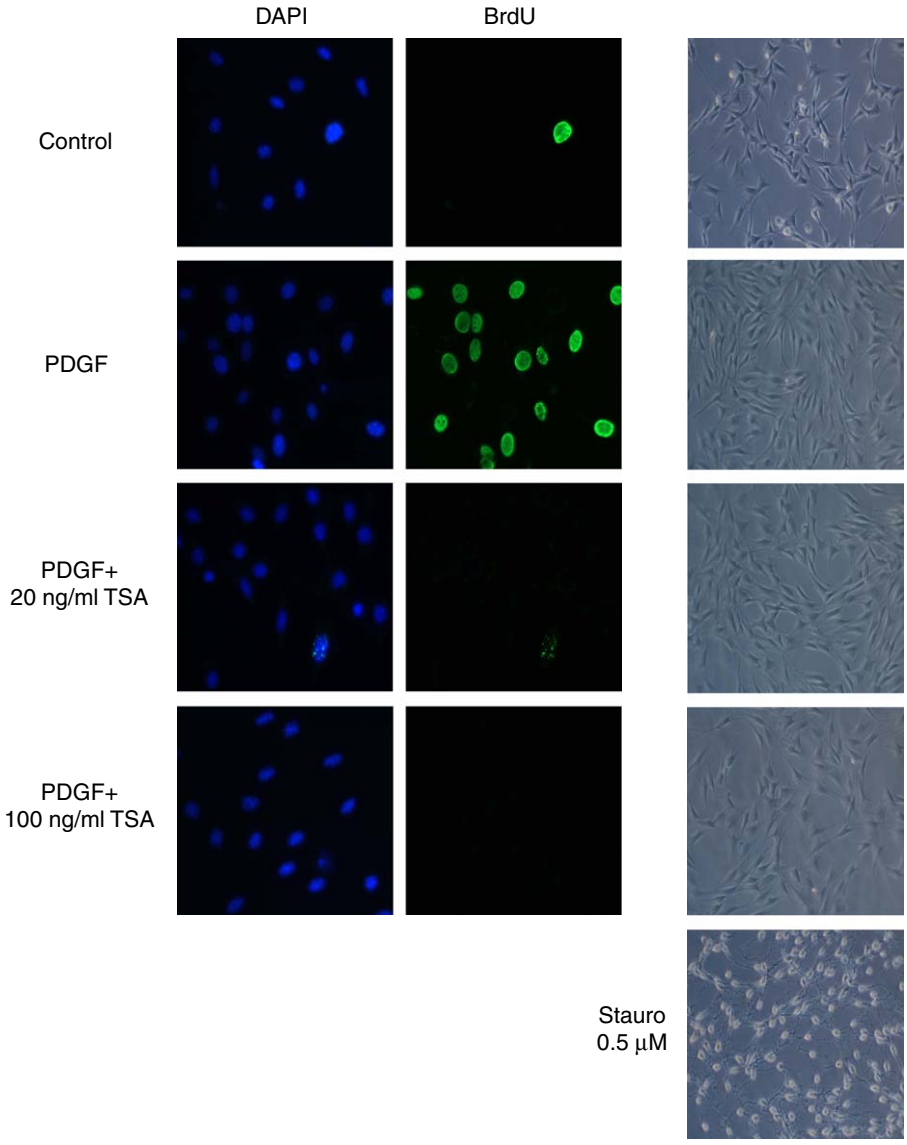

Fig. 8. TSA prevents PDGF-dependent cellular proliferation. NIH3T3 cells were seeded on coverslips. After 24 h, cells were transferred to serum-free medium for 24 h, to arrest them in the G_0/G_1-phase of the cell cycle. NIH3T3 cells were left untreated or pretreated with increasing concentrations of TSA and then stimulated with PDGF for 15 h. Subsequently, cells were incubated with BrdU for 4 h, fixed, and analyzed by immunofluorescence with a specific antibody anti-BrdU (left panels) and for nuclear staining with DAPI (middle panels), as indicated. As a control that TSA treatment was not inducing apoptosis, parallel samples were also analyzed by direct microscopy to ascertain eventual morphological signs of programmed cell death (right panels). As a positive control for apoptosis, NIH3T3 cells were also treated with 0.5 μM staurosporine (Stauro).

STAT proteins in mediating HDAC effects on PDGF transcriptional program. Nonetheless, inhibition of STAT activity by TSA was not correlated to a deficit in STAT3 tyrosine and serine phosphorylation, nucleo-cytoplasmic shuttling, and both in vitro and in vivo DNA-binding activities. TSA also inhibits selected interferon β-stimulated immediate early genes that are activated by STAT1 and STAT2 although, in line with our results, the drug does not affect tyrosine phosphorylation of the transcription factors or their binding to the endogenous *ISG54* promoter [34]. Conversely, TSA prevents the binding of RNA polymerase II to this promoter [34]. Further work will be required to ascertain a role for STAT proteins in HDAC-dependent recruitment of RNA polymerase II to the promoter of specific genes.

Numerous data indicate that the exposure of quiescent cells to PDGF causes the rapid activation of a number of signaling pathways controlling re-initiation of DNA synthesis and cell proliferation [11,29]. We show that one such pathway requires HDAC activity as TSA completely prevents PDGF-dependent cellular proliferation. Intriguingly, Bowman and collaborators recently showed that STAT3-mediated c-Myc expression is required for PDGF-induced mitogenesis [38]. Together, these observations provide support for a requirement for histone deacetylase activity in the control of a STAT-dependent transcriptional program

induced by PDGF, culminating in the increased expression of growth-related genes and, consequently, cellular proliferation.

In contrast to the idea of HDACs as regulators of global chromatin organization, the effects of HDAC inhibitors on gene expression are surprisingly highly selective, leading to modification of the transcription rate of only a limited number of expressed genes [7,8]. As a consequence, there is ongoing evaluation of several HDAC inhibitor compounds in phase I and II clinical trials in a vast array of human tumors (www.clinicaltrials.gov) [8]. Among these drugs, SAHA has already shown significant anticancer activity at doses well tolerated by patients [39].

In the case of PDGF, both solid and hematological malignancies have been identified that present constitutive activation of the signaling pathways controlled by its cognate receptor [3]. The involvement of HDACs in the PDGF-dependent mitogenic transcriptional program and cell proliferation may therefore represent a valid rationale for the use of these drugs in cancers in which deregulated PDGF receptor signaling represents the cause of the tumor or strongly sustains their maintenance through anti-apoptotic and pro-angiogenic processes.

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