UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

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Tesi Sperimentale di Dottorato

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

Coordinatore
Prof. Enrico Vittorio Avvedimento

Candidato
Dott.ssa Annunziata Catania

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Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano"

Tesi di Dottorato in Patologia e Fisiopatologia Molecolare
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Candidato: Dott.ssa Annunziata Catania

Docente Guida: Prof.ssa Maria Stella Carlomagno

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Coordinatore del Corso di Dottorato:
Prof. Enrico Vittorio Avvedimento

Sede Amministrativa:
Università degli Studi di Napoli "Federico II"

Dipartimenti concorrenti:
Biochimica e Biotecnologie Mediche

Collegio dei Docenti

Prof. Enrico Vittorio Avvedimento: Coordinatore del dottorato
Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano", Università di Napoli

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Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli

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Prof. Maria Stella Carlomagno
Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano" Università di Napoli

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Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli

Dott. Guglielmo R.D. Villani
Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli

Dott. Maria Stella Zannini
Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano" Università di Napoli

Prof. Raffaele Zarrilli
Dipartimento di Biologia e Patologia Cellulare e Molecolare "L.Califano" Università di Napoli

Prof. Chiara Zurzolo
Dipartimento di Biologia e Patologia Cellulare e Molecolare "L.Califano" Università di Napoli

1. Introduction

Platelet-derived growth factor (PDGF) is a major mitogen for fibroblasts, smooth muscle cells and other cell types (CH Heldin, B Westermark. Physiol Rev 79 (1999) 1283). 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) (CH Heldin, B Westermark. Physiol Rev 79 (1999) 1283.)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 (SG Devare, et al.; Proc Natl Acad Sci USA 80 (1983) 731), genetic alterations have been characterized which cause constitutive activation of PDGF receptors, autocrine growth stimulation and consequently, human cancer (K Pietras, et al.;. Cancer Cell 3 (2003) 439). Consequently, the 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 (PA Marks, et al.; Nature Reviews Cancer 1 (2001) 194) **(FIG 1-2)**. 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 (DH Kim, et al.; J Biochem Mol Biol 36 (2003) 110). 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 (I Nusinzon, CM Horvath. Sci STKE 2005 296 (2005) re11). Two classes of enzymes,

histone acetyltransferases (HATs) **(TAB.1)** and histone deacetylases (HDACs) **(TAB.2)**, reversibly regulate the extent of such modifications (PA Marks, et al.; Nature Reviews Cancer 1 (2001) 194) **(FIG.3)**.

Different studies have recently demonstrated that histones are not the only proteins under the control of HATs and HDACs (PA Marks, et al.; Nature Reviews Cancer 1 (2001) 194). Thus, substrates for acetylation now include several transcription factors, cytosolic proteins such as Tubulin, and proteins that shuttle from the nucleus to the cytoplasm such as Importin (F McLaughlin, NB La Thangue. Biochem Pharmacol 68 (2004) 1139) **(TAB. 3)** 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 (DH Kim, et al.; J Biochem Mol Biol 36 (2003) 110). Since then, huge efforts has 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), cyclic tetrapeptides such as trapoxin A and benzamides hydroxamic acids such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) (PA Marks, et al.; Nature Reviews Cancer 1 (2001) 194) **(TAB. 4)**. As expected from their ability to stimulate gene expression by acting on histones, HDAC inhibitors induce the levels of 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 (I Nusinzon, CM Horvath. Sci STKE 2005 296 (2005) re11). Still, more recently it has become clear that they are also able to inhibit the expression of tumor activators such as VEGF (DH Kim, et al.; J Biochem Mol Biol 36 (2003) 110), c-Myc, Bcl-X_L and HIF-1 (MJ Peart, et al.; Proc Natl

Acad Sci USA 102 (2005) 3697), suggesting for these drugs a mechanism of action more complex then the mere effect on histone acetylation.

2. Materials and methods

2.1. Reagents

Human recombinant PDGF (Intergen) was used at a final concentration of 12.5 ng ml–1. The HDAC inhibitors SAHA and TSA (Biomol) were added to the cells 30 min before stimulation at the indicated concentrations. The Stat responsive element (x4) luciferase vector (pStat-Luc) was kindly provided by J.E. Jr. Darnell (JF Bromberg, et al.; Mol Cell Biol 18 (1998) 2553). 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.
NIH 3T3 fibroblasts were maintained at 37°C in 5% CO2 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).

2.2. Northern blot analysis

After 24 hrs of starvation, NIH3T3 cells were stimulated with 12.5 ng ml-1 PDGF for various times in absence or after pre-treatment with increasing concentration of TSA. Samples were
then processed as previously described (M Chiariello, et al.; Nat Cell Biol 3 (2001) 580). 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 (pCEFLP-VEGF). As *bcl-XL* probe, we used a 500-bp Eco RI fragment

from the *bcl-XL* cDNA (pcDNA4/TO-Bcl-XL, kindly provided by I. Iaccarino). The RNA membranes were pre-hybridized for 2 hrs in hybridization solution (ExpressHyb, Clontech) at 70 °C. The 32Plabeled probe for the human c-*myc* gene was added to the blots and hybridized for another 16 hrs at 60 °C. The 32P-labeled probes of the human *VEGF* and *bcl-XL* genes were added to the blots and hybridized for another 16 hrs at 68 °C. The blots were washed in accordance with manufacturer's specifications of hybridization solution (ExpressHyb, Clontech). Accuracy of RNA loading and transfer was confirmed by fluorescence under ultraviolet light after staining with ethidium bromide.

2.3. 5-Bromo-2'deoxy-uridine (BrdU) assay

NIH3T3 cells were starved for 24 hrs, stimulated with 12.5 ng ml-1 PDGF for 15 hrs in absence or after pre-treatment for 30 min with increasing concentration of TSA, before incubation with BrdU (10 mmol) for 4 hrs. 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.

2.4. Electrophoretic Mobility Shift Assays (EMSA)

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 concentration of TSA. Samples were then processed as previously described (C Iavarone, et al.; J Biol Chem 278 (2003) 50024). Complementary synthetic oligonucleotides containing the STAT3 consensus sequence from Santa Cruz Biotechnology were labeled with [γ-32P]-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, 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.

2.5. 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). EMSA and Western blot analysis were performed using rabbit polyclonal antibodies against STAT3-[pSer727] (BIOSOURCE), STAT3-[pTyr705] (Cell Signaling Technology), H3 (Novous Biologicals), STAT3 and Acetyl-Histone H3 (UPSTATE), STAT3 C20-X (Santa Cruz Biotechnology).

2.6. 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 of 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 hrs 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 20/20n luminometer (Turner BioSystems).

2.7. Immunofluorescences

NIH3T3 cells were starved for 24 hrs, stimulated with 12.5 ng ml-1 PDGF for 1 hr in absence or after pre-treatment for 30 min with increasing concentration of TSA. The cells were fixed for 10 min in 2% paraformaldeide-1% sucrose solution at room temperature. Incubation with anti-STAT3 antibodies (Upstate Biotecnology) was preformed 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 hr at room temperature. The slides were mounted in Gel-mount (Biomeda Corp.) and examined with a Zeiss Axiophot photomicroscope equipped with epifluorescence.

3. Results

3.1. 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 (MJ Peartet al.;. Proc Natl Acad Sci USA 102 (2005) 3697; W Wharton, et al.; J Biol Chem 275 (2000) 33981; XD Zhang, et al.; Mol Cancer Ther 3 (2004) 425). Indeed, differently from what 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 (I Nusinzon, CM Horvath. Proc Natl Acad Sci USA 100 (2003) 14742). As an approach to examine, in NIH3T3 cells, the role of acetylation in PDGF-dependent transcriptional activity, we took advantage of the availability of pharmacological inhibitors of HDACs (PA Marks, et al.; Nature Reviews Cancer 1 (2001) 194). In particular, trichostatin A (TSA)

potently and specifically inhibits HDACs causing an accumulation of acetylated histone species in a variety of mammalian cell lines (M Yoshida, et al.; J Biol Chem 265 (1990) 17174).

We first sought to confirm the ability of this compound to affect histone acetylation in our cellular system, NIH3T3 murine fibroblasts. As shown in **figure 5A**, TSA strongly induced, in a dose-dependent manner, histone H3 acetylation, after 18 hrs 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 and* $bcl\text{-}X_L$. Cells were starved for 24 hrs and then stimulated with PDGF for up to 7 hrs. Northern blot analysis of the extracted total RNA showed an increase in the levels of the three genes, although at different time-points after PDGF stimulation. Indeed, while as expected (M Chiariello, et al.;. Nat Cell Biol 3 (2001) 580) PDGF caused a peak of c-*myc* mRNA after one hour of treatment **(Fig. 5B)**, such increase was delayed for *VEGF* (3 hrs) **(Fig. 5C)** and $bcl\text{-}X_L$ (7 hrs) **(Fig. 5D)**. Still, in all cases such increase was strongly inhibited by 30 min pretreatment with increasing concentrations of TSA, establishing a role for acetylation in the early and late gene expression processes controlled by PDGF **(Fig. 5E, 5F and 5G)**. Also, to confirm that inhibition of gene expression was the result of the activity of TSA on HDACs, a second HDAC inhibitor, SAHA, was used in a similar experiment, to assess its ability to interfere with PDGF-dependent stimulation of c-*myc* expression. As shown in **figure 5H**, SAHA was also very efficient in blocking PDGF-induced c-*myc* expression, therefore strongly supporting that the effects observed for TSA on gene expression are dependent on its histone deacetylase activity. Altogether, these results therefore show that deacetylase activity is important for both the early and late PDGF-dependent transcriptional program.

3.2. 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 latently residing in the cytoplasm until specific cell-surface receptors activate them (DE Levy, JE Darnell. Nat Rev Mol Cell Biol 3 (2002) 651). Although originally identified as mediators of cytokine signaling, STAT proteins are also activated by polypeptide growth factors such as PDGF and epidermal growth factor (EGF) (Z Zhong, et al.; Science 264 (1994) 95; YZ Wang, et al.; Oncogene 19 (2000) 2075). STAT proteins are involved in the regulated expression of numerous genes underlying diverse cellular processes ranging from the immune response to antiviral protection, apoptosis, proliferation, differentiation and cell survival (DE Levy, JE Darnell. Nat Rev Mol Cell Biol 3 (2002) 651). Among the regulated genes, expression of growth-controlling genes such as *cyclin D1*, *bcl-*X_L, *VEGF* and c-*myc* has been correlated to STAT activity (T Fukada, et al.; Embo J 17 (1998) 6670; M Socolovsky, et al.; Cell 98 (1999) 181). Aberrant STAT signaling may also participate in development and progression of human cancers (DE Levy, CK Lee. J Clin Invest 109 (2002) 1143) (**TAB.5**). 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 (DE Levy, CK Lee. J Clin Invest 109 (2002) 1143).

In search for a mechanism that could explain the ability of TSA to inhibit PDGF-dependent gene expression, we noticed that all investigated genes are under the control of STAT transcription factors (M Funamoto, et al.; J Biol Chem 275 (2000) 10561; R Catlett-Falcone, et al.; Immunity 10 (1999) 105; N Kiuchi, et al.; J Exp Med 189 (1999) 63). As numerous recent reports point to a positive role for HDACs in cytokine- and STAT-dependent gene regulation(I

Nusinzon, CM Horvath. Sci STKE 2005 296 (2005) re11), 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 (JF Bromberg, et al.; Mol Cell Biol 18 (1998) 2553). NIH3T3 cells were therefore transiently transfected with this reporter, left untreated or treated with increasing concentrations of TSA and PDGF (6 hrs), alone or in combination **(Fig. 6A)**. While PDGF strongly induced STAT activity in these cells, TSA almost abolished such response **(Fig. 6A)**, 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 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 hrs), alone or in combination **(Fig. 6B)**. Again, while PDGF strongly induced STAT3 activity in these cells, TSA almost abolished such response **(Fig. 6B)**, therefore suggesting that HDAC activity is required for optimal PDGF-dependent activation of STAT3.

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

Activation of STAT proteins requires phosphorylation of cytosolic STAT monomers on a single tyrosine residue at their C-terminus (DE Levy, JE Darnell. Nat Rev Mol Cell Biol 3 (2002) 651) **(FIG.7)**. 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 (DE Levy, JE Darnell. Nat Rev Mol Cell Biol 3 (2002) 651) **(FIG.8)**. Some STATs also require phosphorylation on a conserved serine residue for maximal transcriptional activation (WJ Leonard, JJ O'Shea. Annual Review of Immunology 16 (1998) 293).

To determine whether HDAC inhibition alters PDGF-stimulated tyrosine or serine phosphorylation of STAT3, we performed both western blot analysis with 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 **figure 9**, 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. 10)**. 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 (T Meyer, et al.; Exp Cell Res 272 (2002) 45).

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. 11A)**, 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 (farthest migrating complexes) and STAT1/STAT3 hetero-dimers (complexes with the intermediate mobility) (HB Sadowski, et al.; Science 261 (1993) 1739) **(Fig. 5A)**. 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. 5B)**. 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. 5C)**. As shown in **figure 5D**, 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. 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.

3.4. Inhibition of HDAC activity prevents PDGF-dependent cellular proliferation

HDAC inhibitors have repeatedly demonstrated their efficacy to arrest cellular growth of multiple cell lines (PA Marks, et al.; Nature Reviews Cancer 1 (2001) 194; DH Kim, et al.; J Biochem Mol Biol 36 (2003) 110). 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 concentration of TSA. Cells were first starved for 24 hrs 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 hrs, a timeframe in which NIH3T3 cells enter S-phase and duplicate their DNA (M Chiariello, et al.; Biochem J 349 (2000) 869). As shown in **Fig. 6**, the addition of TSA was able to completely inhibit S-phase progression of stimulated NIH3T3 cells, even at the lowest concentration used (50 μM). TSA was therefore a strong inhibitor of the early phases of cell cycle progression of NIH3T3 cells stimulated with PDGF.

4. Discussion

Although many different genetic defects exist in human tumors, they frequently seem to converge on a more limited number of signal transduction pathways often controlling the expression of a certain number of cancer promoting genes. The possibility to modulate the expression of such genes has therefore become a rationale 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 (F McLaughlin, NB La Thangue. Biochem Pharmacol 68 (2004) 1139). Though, in this respect, it is important to note that several non-histonic proteins, among which different transcription factors, are direct substrates of acetylation and, in turn, of HDACs (F McLaughlin, NB La Thangue. Biochem Pharmacol 68 (2004) 1139).

By using TSA, one of the first HDAC inhibitors identified, 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 are under the control of these transcription factors (M Funamoto, et al.; J Biol Chem 275 (2000) 10561; R Catlett-Falcone, et al.;.

Immunità 10 (1999) 105; N Kiuchi, et al.; J Exp Med 189 (1999) 63), suggesting 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 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 (L Klampfer, et al.; Mol Cancer Res 1 (2003) 855; L Klampfer, et al.; J Biol Chem 279 (2004) 36680), other papers indicate that these drugs have no direct effect on nuclear translocation, DNA-binding activity and tyrosine and serine phosphorylation of STAT proteins (I Nusinzon, CM Horvath. Proc Natl Acad Sci USA 100 (2003) 14742; S Sakamoto, et al.;. J Biol Chem 279 (2004) 40362; HM Chang, ET AL.; Proc Natl Acad Sci USA 101 (2004) 9578). Complicating even more this story, recent data report that, upon cytokine stimulation, STAT3 undergo acetylation of a single amino acid residue providing an alternative mechanism for its activation (ZL Yuan, et al.; Science 307 (2005) 269; R Wang, et al.; J Biol Chem 280 (2005) 11528), 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 STAT proteins in mediating HDACs 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 DNA-binding activity. 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 (S Sakamoto, et al.; J Biol Chem 279

(2004) 40362). Conversely, TSA prevents the binding of RNA polymerase II to this promoter (S Sakamoto, et al.; J Biol Chem 279 (2004) 40362). Further work will be required to ascertain a role for STAT proteins in HDACs-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 (M Chiariello, et al.; Nat Cell Biol 3 (2001) 580; M Chiariello, et al.; Biochem J 349 (2000) 869). 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 (T Bowman et al.; Proc Natl Acad Sci USA 98 (2001) 7319).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 (F McLaughlin, NB La Thangue. Biochem Pharmacol 68 (2004) 1139). 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) (F McLaughlin, NB La Thangue. Biochem Pharmacol 68 (2004) 1139). Among these drugs, SAHA has already shown significant anticancer activity at doses well tolerated by patients (WK Kelly, et al.; Clin Cancer Res 9 (2003) 3578).

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 (K Pietras, et al.; Cancer Cell 3 (2003) 439).

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 sustain their maintenance through anti-apoptotic and pro-angiogenic processes.

References

Bowman T, MA Broome, D Sinibaldi, W Wharton, WJ Pledger, JM Sedivy, R Irby, T Yeatman, SA Courtneidge, R Jove. Proc Natl Acad Sci USA 98 (2001) 7319.

Bromberg JF, CM Horvath, D Besser, WW Lathem, JE, Jr. Darnell Mol Cell Biol 18 (1998) 2553.

Catlett-Falcone R, TH Landowski, MM Oshiro, J Turkson, A Levitzki, R Savino, G Ciliberto, L Moscinski, JL Fernandez-Luna, G Nunez, WS Dalton, R Jove. Immunità 10 (1999) 105.

Chang HM, M Paulson, M Holko, CM Rice, BRG Williams, I Marie, DE Levy. Proc Natl Acad Sci USA 101 (2004) 9578.

Chiariello M, E Gomez, JS Gutkind. Biochem J 349 (2000) 869.

Chiariello M, MJ Marinissen, JS Gutkind. Nat Cell Biol 3 (2001) 580.

Devare SG, EP Reddy, JD Law, KC Robbins, SA Aaronson. Proc Natl Acad Sci USA 80 (1983) 731.

Fukada T, T Ohtani, Y Yoshida, T Shirogane, K Nishida, K Nakajima, M Hibi, T Hirano. Embo 17 (1998) 6670.

Funamoto M, Y Fujio, K Kunisada, S Negoro, E Tone, T Osugi, H Hirota, M Izumi, K Yoshizaki, K Walsh, T Kishimoto, K Yamauchi-Takihara. J Biol Chem 275 (2000) 10561.

Heldin CH, B Westermark. Physiol Rev 79 (1999) 1283.

Iavarone C, A Catania, MJ Marinissen, R Visconti, M Acunzo, C Tarantino, MS Carlomagno, CB Bruni, JS Gutkind, M Chiariello. J Biol Chem 278 (2003) 50024.

Kelly WK, VM Richon, O O'Connor, T Curley, B MacGregor-Curtelli, W Tong, M Klang, L Schwartz, S Richardson, E Rosa, M Drobnjak, C Cordon-Cordo, JH Chiao, R Rifkind, PA Marks, H Scher. Clin Cancer Res 9 (2003) 3578.

Kim DH, M Kim, HJ Kwon. J Biochem Mol Biol 36 (2003) 110.

Kiuchi N, K Nakajima, M Ichiba, T Fukada, M Narimatsu, K Mizuno, M Hibi, T Hirano. J Exp Med 189 (1999) 63.

Klampfer L, J Huang, T Sasazuki, S Shirasawa, L Augenlicht. J Biol Chem 279 (2004) 36680.

Klampfer L, J Huang, T Sasazuki, S Shirasawa, L Augenlicht. Mol Cancer Res 1 (2003) 855.

Leonard WJ, JJ O'Shea. Annual Review of Immunology 16 (1998) 293.

Levy DE, CK Lee. J Clin Invest 109 (2002) 1143.

Levy DE, JE Darnell. Nat Rev Mol Cell Biol 3 (2002) 651.

Marks PA, RA Rifkind, VM Richon, R Breslow, T Miller, WK Kelly. Nature Reviews Cancer 1 (2001) 194.

McLaughlin F, NB La Thangue. Biochem Pharmacol 68 (2004) 1139.

Meyer T, K Gavenis, U Vinkemeier. Exp Cell Res 272 (2002) 45.

Nusinzon I, CM Horvath. Proc Natl Acad Sci USA 100 (2003) 14742.

Nusinzon I, CM Horvath. Sci STKE 2005 296 (2005) re11.

Peart MJ, GK Smyth, RK van Laar, DD Bowtell, VM Richon, PA Marks, AJ Holloway, RW Johnstone. Proc Natl Acad Sci USA 102 (2005) 3697.

Pietras K, T Sjoblom, K Rubin, CH Heldin, A Ostman. Cancer Cell 3 (2003) 439.

Sadowski HB, K Shuai, JE, Jr. Darnell, MZ Gilman. Science 261 (1993) 1739.

Sakamoto S, R Potla, AC Larner. J Biol Chem 279 (2004) 40362.

Socolovsky M, AEJ Fallon, S Wang, C Brugnara, HF Lodish. Cell 98 (1999) 181.

Wang R, P Cherukuri, J Luo. J Biol Chem 280 (2005) 11528.

Wang YZ, W Wharton, R Garcia, A Kraker, R Jove, WU Pledger. Oncogene 19 (2000) 2075.

Wharton W, J Savell, WD Cress, E Seto, WJ Pledger. J Biol Chem 275 (2000) 33981.

Yoshida M, M Kijima, M Akita, T Beppu. J Biol Chem 265 (1990) 17174.

Yuan ZL, YJ Guan, D Chatterjee, YE Chin. Science 307 (2005) 269.

Zhang XD, SK Gillespie, JM Borrow, P Hersey. Mol Cancer Ther 3 (2004) 425.

Zhong Z, Z Wen, JE, Jr. Darnell Science 264 (1994) 95.

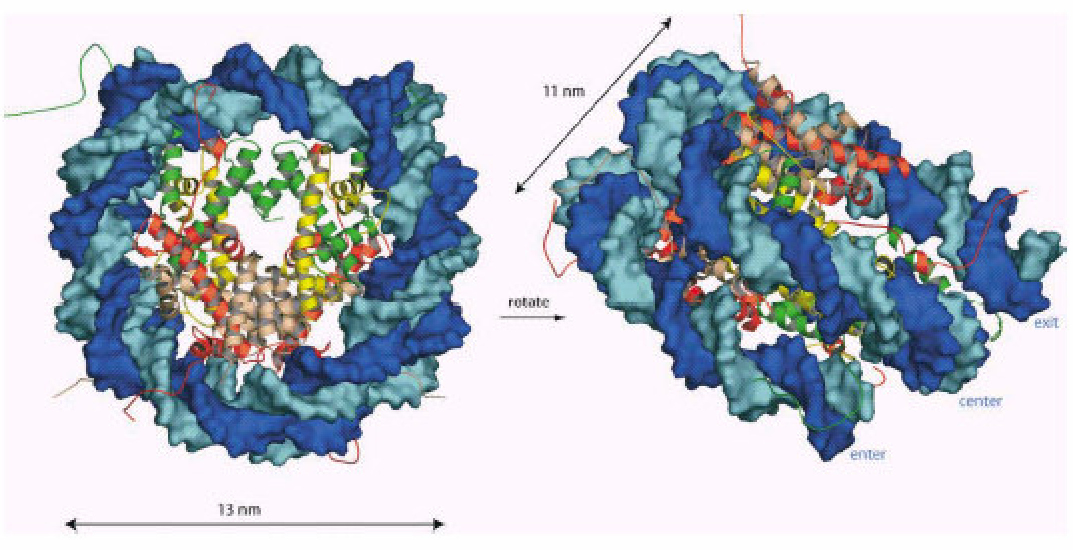

FIG. 1. The Atomic Structure of the Nucleosome Core Particle
Each strand of DNA is shown in different shade of blue. The DNA makes 1.7 turns around the histone octamer to form an overall particle with a disk-like structure.
Sepideh Khorasanizadeh. Cell 2004, 116 :259–272

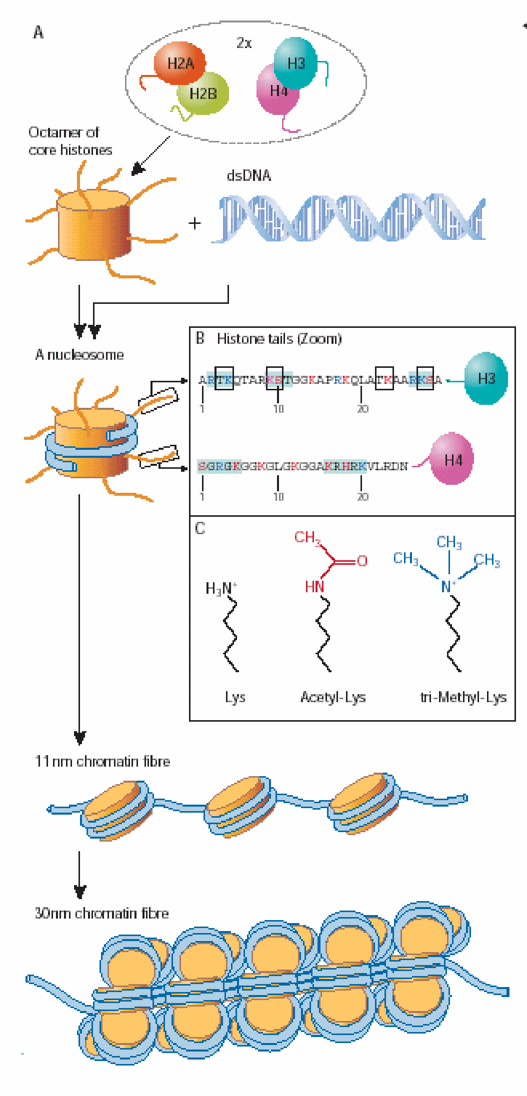

FIG. 2 | The wide range of histone modifications. (**A**) Chromatin is formed by nucleosome subunits comprising an octameric core of histones around which ~1.8 superhelical turns of DNA are wrapped.DNA binds to the positively charged histone surfaces through H-bonds and electrostatic interactions. In the 11-nm chromatin fibre, successive nucleosomes are separated by 10–80 base pairs of linker DNA.Histone H1, which binds to nucleosomes and adjacent linker DNA, can mediate further condensation into the 30-nm chromatin fibre.

(**B**) The amino-termini of histones H3 and H4 protrude from the nucleosome core ad contain dense clusters of modifiable residues.Residues are coloured on welldocumented modification sites,with red indicating where acetylation or phosphorylation increase acidity and blue indicating methylation.Note that H3 Lys 9 (pink) can be either acetylated or methylated.Pale green shading highlights putative ‘modification cassettes’ and boxes indicate potential ‘methyl–phos binary switches’. Further possible modifications are listed elsewhere (Felsenfeld & Groudine, 2003; Fischle *et al*, 2003a).

(**C**) Histone modifications create new chemical environments. For example, lysine acetylation (red) neutralizes the positive charge of the Næ group and introduces the carbonyl oxygen,which is a potential H-bond acceptor.Lysine methylation (blue) increases both
hydrophobicity and the cationic nature of the Næ group.Depending on the modifying enzyme, lysines can be mono-, di- or tri-methylated.

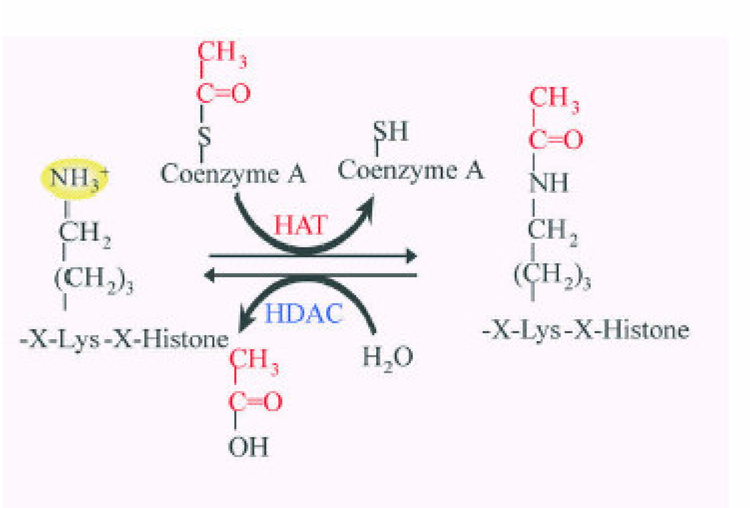

FIG. 3. Role of HAT and HDAC in transcriptional regulation. Histone modification by HAT and HDAC.

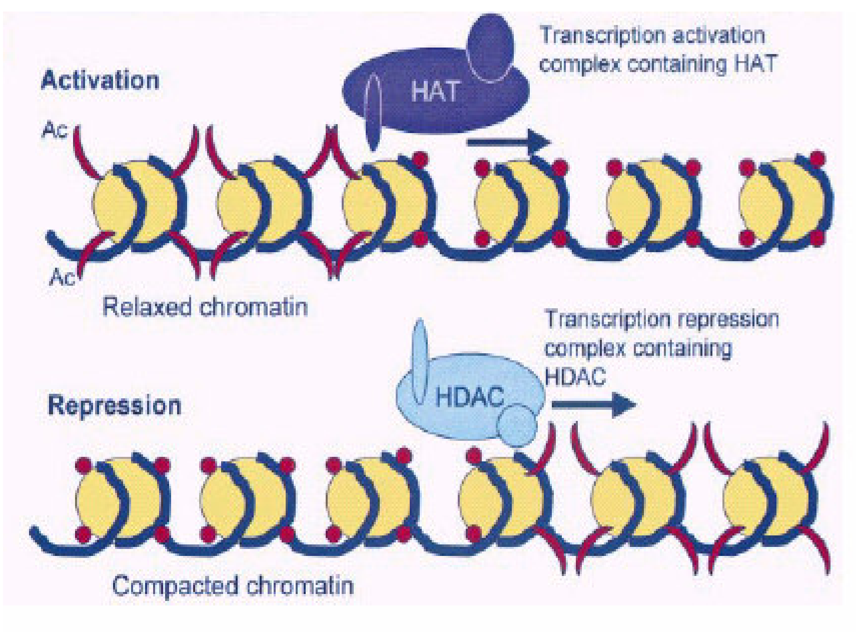

FIG. 4. Transcriptional repression and activation in chromatin. Yellow circles represent core histone octamers ; in the upper panel, acetylated histone tails (dark red) are depicted emerging from the octamer. DNA is purple, and the solid black arrow represents complex movement. Both histone acetyltransferase (HAT ; activation) and HDAC (repression) require several cofactors (for DNA binding, for recruitment of the complex, for remodelling of the DNA helix to reduce the accessibility of transcription factors) for their activity

TABLE 1. Summary of known and putative HATs

HAT	Organisms known to contain the HAT	Known transcription-related functions/effects	HAT activity demonstrated in vitro[b]	Histone specificity of recombinant enzyme in vitro[a,b]	Known native HAT complexes and nucleosomal histone specificities in vitro
GNAT super-family					
Hat1	Various (yeast to humans)	None (histone deposition-related B-type HAT)	Yes	**H4**	Yeast HAT-B, HAT-A3 (no nucleosome acetylation)
Gcn5	Various (yeast to humans)	Coactivator (adaptor)	Yes	**H3**/H4	Yeast ADA, SAGA (**H3**/H2B); human GCN5 complex, STAGA, TFTC (**H3**)
PCAF	Humans, mice	Coactivator	Yes	**H3**/H4	Human PCAF complex (**H3**/weak H4)
Elp3	Yeast	Transcript elongation	Yes	ND*	Elongator, polymerase II holoenzyme (**H3**/weak H4)
Hpa2	Yeast	Unknown	Yes	**H3**/H4	
MYST family					
Sas2	Yeast	Silencing	ND		
Sas3	Yeast	Silencing	Yes	**H3**/**H4**/H2A	NuA3[c] (**H3**)
Esa1	Yeast	Cell cycle progression	Yes	**H4**/H3/H2A	NuA4 (**H4**/H2A)
MOF	*Drosophila*	Dosage compensation	Yes	**H4**/H3/H2A	MSL complex (**H4**)
Tip60	Humans	HIV Tat interaction	Yes	**H4**/H3/H2A	Tip60 complex
MOZ	Humans	Leukemogenesis, upon chromosomal translocation	ND		
MORF	Humans	Unknown (strong homology to MOZ)	Yes	**H4**/H3/H2A	
HBO1	Humans	ORC interaction	Yes*	ND*	HBO1 complex
p300/CBP	Various multicellular	Global coactivator	Yes	**H2A**/**H2B**/**H3**/**H4**	
Nuclear receptor coactivators		Nuclear receptor coactivators (transcriptional response to hormone signals)			
SRC-1	Humans, mice		Yes	**H3**/H4	
ACTR	Humans, mice		Yes	**H3**/**H4**	
TIF2	Humans, mice		ND		
$TAF_{II}250$	Various (yeast to humans)	TBP-associated factor	Yes	**H3**/H4	TFIID
TFIIIC		RNA polymerase III transcription initiation			TFIIIC (**H2A**/**H3**/**H4**)
TFIIIC220	Humans		Yes*	ND	
TFIIIC110	Humans		Yes	ND	
TFIIIC90	Humans		Yes	**H3**	

Table 1 *a* Histones that are the primary in vitro substrates for a given HAT are bold; other histones listed are acetylated weakly or in a secondary manner.
b Asterisks indicate proteins for which HAT activity has been suggested indirectly or demonstrated in an incomplete manner. Elp3 can acetylate all four histones but has only been tested with them individually in in-gel assays. The HAT function of HBO1 has primarily been shown by the in vitro free histone **H3/**H4-acetylating activity of a purified human complex containing it, although recombinant GST-HBO1 (and the complex) did weakly acetylate nucleosomes. Finally, TFIIIC220 was identified as a HAT only in in-gel assays, and its activity has not been confirmed by recombinant protein studies as of this writing. ND, not determined.*c* S. John and J. L. Workman, unpublished result.

Table 2. Characteristics of histone deacetylases

	Class I	Class IIa	Class IIb	Class III
Yeast HDAC	RPD3	HDA1	HDA1	SIR2
Human HDAC	HDAC 1, 2, 3, 8, 11	HDAC 4, 5, 7, 9	HDAC 6, 10	SIRT 1, 2, 3, 4, 5, 6, 7
Distribution	Ubiquitous	Tissue-restricted	Tissue-restricted	?
Localization	Nuclear	Nucl./cytopl.	Nucl./cytopl.	Nucl./cytopl./Mitochondrial
Targets	Histones p53 (HDAC1), NF-kB (HDAC3)	Histones	Histones, Tubulin	Histones, Tubulin (SIRT2), p53 (SIRT1), TAF(I)68 (SIRT1)
Co-factors	Zn^+	Zn^+	Zn^+	NAD^+
Inhibitor Sensitivity	S	S	S	NS/ND

Table 3. Acetylated protein substrates of HDACs

Histones	H2a, H2b, H3, H4
Transcription factors	TAT, p53, p73, TCF, GATA-1, RelA, C-Jun, E2F, EKLF, NF-Y, HMGI (Y), NF-kB
Nuclear import factors	Importin α, Reh1
Cytoskeleton proteins	αTubulin
Chaperon proteins	HSP90

Group and structure	Compounds	*In vitro* IC_{50} range
Hydroxamic acids	TSA [8,24,26,27,32,35,64,79,85,86,101,102,107,108,112,116]	nM
	Suberoyl anilide bishydroxamide (SAHA) [8,10,32,85,86,112]	μM
	M-carboxycinnamic acid bishydroxamide (CBHA) [32,103,105]	μM
	Scriptaid [32,85,86]	μM
	Pyroxamide [32,98]	μM
	Oxamflatin [32,84–86,112]	nM
Short-chain fatty acids	Butyrate [27,30,32,35,79,85,86,102,107,112,116]	mM
	Phenylbutyrate [8,9,25,85,88–90,117]	mM
	Valproic acid [26,32]	mM
Cyclic tetrapeptides/epoxides	Trapoxin [32,33,85,86,112]	nM
	HC-toxin [116]	nM
	Chlamydocin [118]	nM
	Depudesin [32,85,86,111,112]	μM
	Apicidine [30,32,85,86,100,112]	nM–μM
	Depsipeptide (FK228) [31,32,85,86,92,112,122,123]	nM
Benzamides	*N*-acetyldinaline (CI-994) [32,85–87,112,121]	μM
	MS-275 [32,85,86,110,112]	μM

Table 4 Shown are the features of four groups of HDACi, including in vitro efficiency of the members, and general structure. Oxamflatin, apicidine and depsipeptide (FK228) are cyclic hydroxamic acid-containing tetrapeptides, i.e. hybrids between hydroxamic acids and cyclic tetrapeptides. They are grouped in accordance with their major feature. The cyclic tetrapeptides/epoxides form a heterogeneous group of compounds with a high degree of overlap, indicating that many of the members have both features.

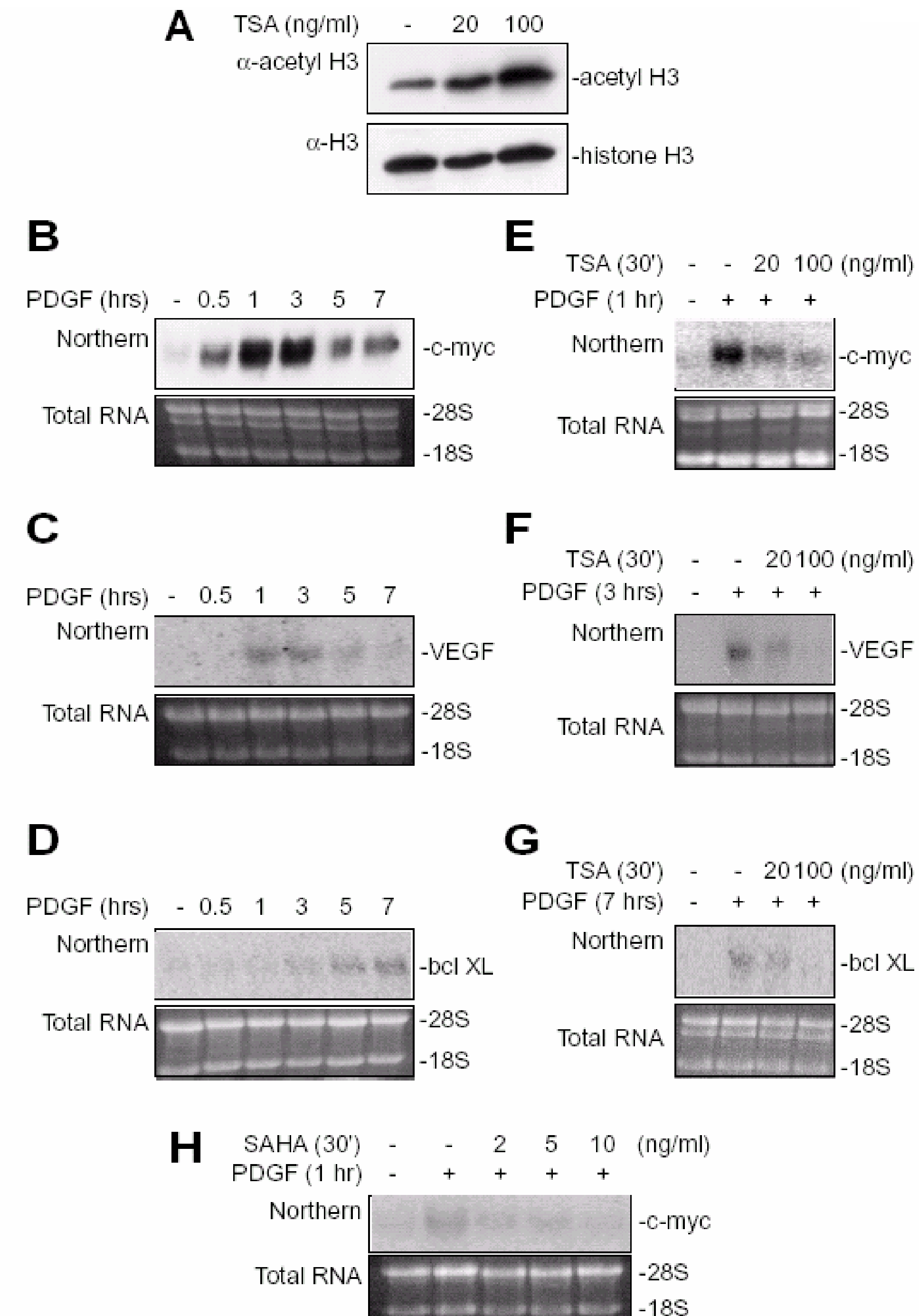

FIG. 5. HDAC inhibitors prevent PDGF-dependent expression of different growth-promoting genes. (A) TSA induces histone H3 acetylation. NIH3T3 cells were starved for 24 hrs and pretreated or not with increasing concentrations of TSA for 15 hrs. Nuclear extracts were then assayed by Western blot, using the specific anti-acetyl-Histone H3 (upper panel) and anti-H3 (bottom panel) antibodies. (B) Analysis of c-*myc* mRNA expression in NIH3T3 cells stimulated for the indicated durations with PDGF. (C) Analysis of *VEGF* mRNA expression in NIH3T3 cells stimulated for the indicated durations with PDGF. (D) Analysis of *bcl-*X_L mRNA expression in NIH3T3 cells stimulated for the indicated durations with PDGF. (E) Analysis of c-*myc* mRNA in NIH3T3 cells pretreated with increasing concentrations of the specific HDAC inhibitor, TSA, and then stimulated for 1 hr with PDGF. (F) Analysis of *VEGF* mRNA in NIH3T3 cells pretreated with increasing concentrations of TSA and then stimulated for 3 hrs with PDGF. (G) Analysis of *bcl-*X_L mRNA in NIH3T3 cells pretreated with increasing concentrations of TSA and then stimulated for 7 hrs with PDGF. (H) Analysis of c-*myc* mRNA in NIH3T3 cells pretreated with increasing concentrations of the specific HDAC inhibitor, SAHA, and then stimulated for 1 hr with PDGF. -, no treatment.

Activation of STATs in human cancers	
Tumour type	**Activated STAT**
Blood tumours	
Multiple myeloma	STAT1, STAT3
Leukaemias:	
HTLV-I-dependent	STAT3, STAT5
Erythroleukaemia	STAT1, STAT5
Acute myelogenous leukaemia (AML)	STAT1, STAT3, STAT5
Chronic myelogenous leukaemia (CML)	STAT5
Large granular lymphocyte leukaemia (LGL)	STAT3
Lymphomas:	
EBV-related/Burkitt's	STAT3
Mycosis fungoides	STAT3
Cutaneous T-cell lymphoma	STAT3
Non-Hodgkins lymphoma (NHL)	STAT3
Anaplastic large-cell lymphoma (ALCL)	STAT3
Solid tumours	
Breast cancer	STAT1, STAT3, STAT5
Head and neck cancer	STAT1, STAT3, STAT5
Melanoma	STAT3
Ovarian cancer	STAT3
Lung cancer	STAT3
Pancreatic cancer	STAT3
Prostate cancer	STAT3

Table 5

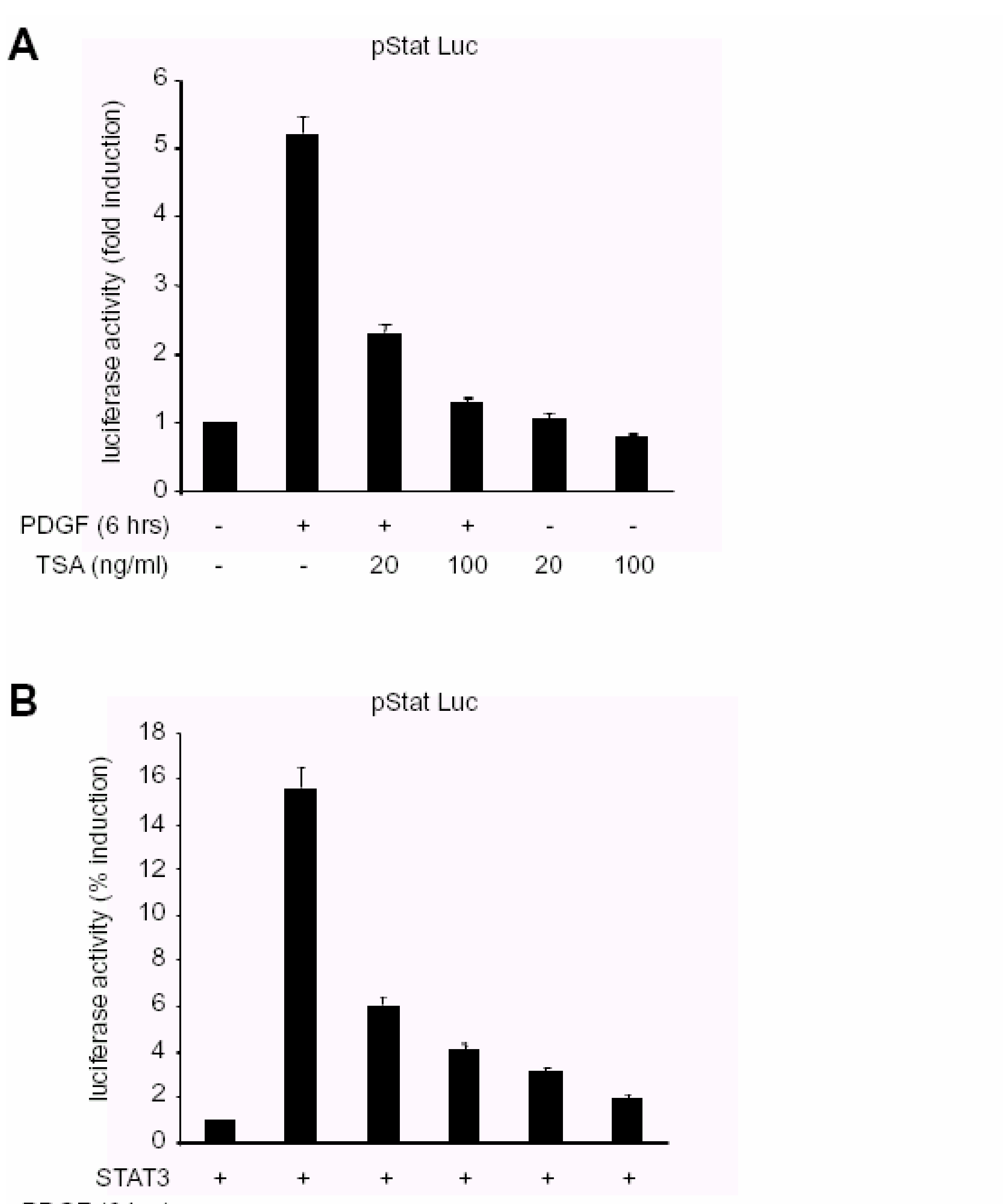

FIG. 6. TSA inhibits STAT transcriptional activity induced by PDGF. (A) NIH3T3 cells were transfected with a STAT-responsive luciferase construct, pStat-Luc (200 ng). The day after transfection, cells were left untreated or treated with combinations of increasing concentrations of TSA and PDGF (6 hrs),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

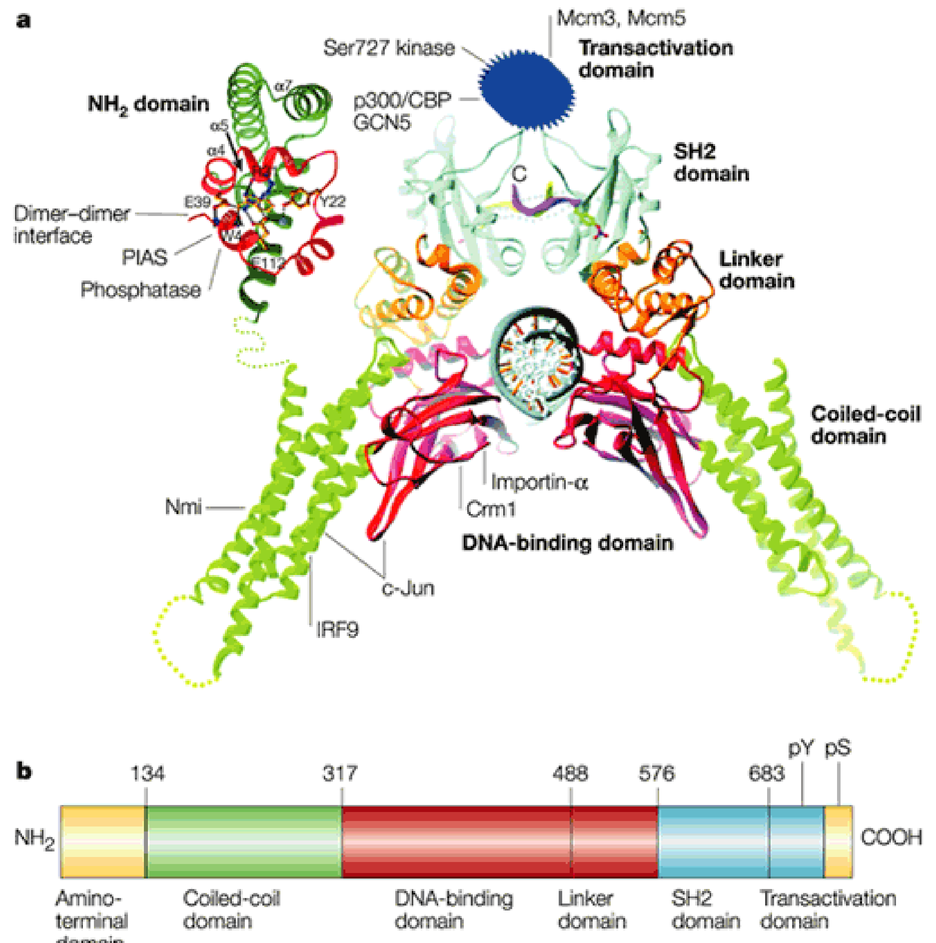

FIG. 7 | STAT domain structure and protein binding sites. **a** | The core structure (amino acids 130–712) shows binding of a STAT1 dimer to DNA and the location of binding sites of various proteins in various domains. The amino-terminal structure, the placement of which in the intact structure is undefined, also interacts with various partners, as does the carboxy-terminal transactivation domain, the structure of which is unknown. Modified with permission from Ref. 36 © 1998 American Association for the Advancement of Science, and from Ref. 161 © 1998 Elsevier Science Ltd. CBP, CREB binding protein; IRF, interferon regulatory factor; Mcm, minichromosome maintenance; Nmi, N-Myc interactor; PIAS, protein inhibitor of activated STAT. **b** | STAT structure. STAT, signal transducer and activator of transcription. SH2, Src-homology-2 domain.

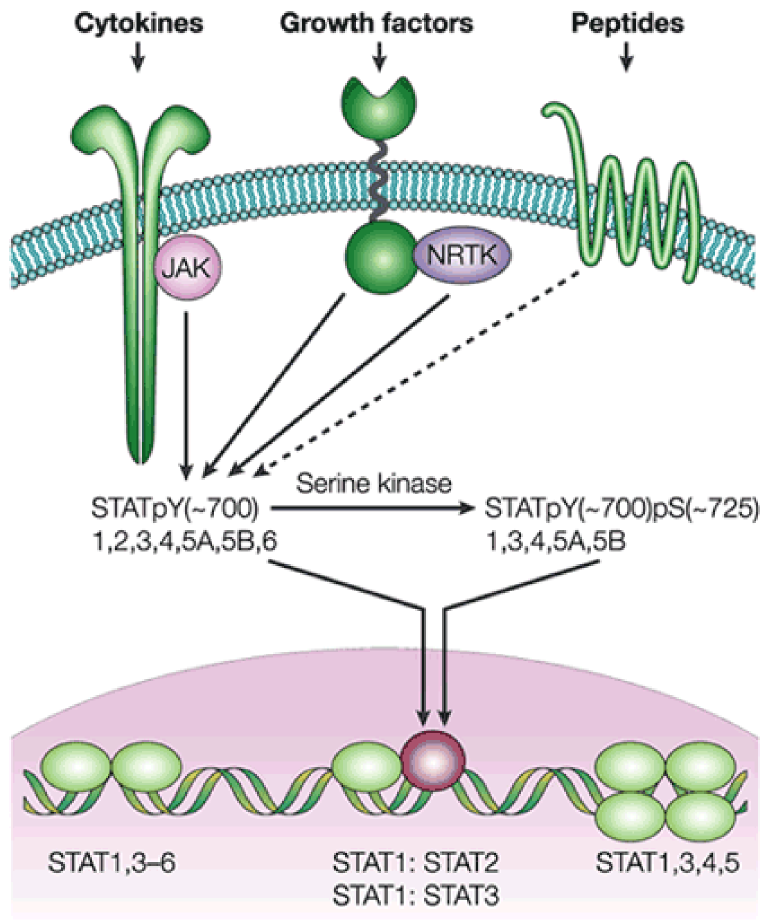

FIG. 8. | Variations in mechanisms of STAT activation

Tyrosine phosphorylation of signal transducers and activators of transcription (STAT) proteins at or around residue 700 occurs in response to cytokine receptors through Janus kinases (JAKs). However, at least several dozen receptors with intrinsic tyrosine kinase activity (RTKs), such as those for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), seem to be able to mediate the activation of STAT proteins. Apparently, this activation can be direct (as in the case of STAT1 activation by PDGF receptor) or indirect. The latter case involves the recruitment of complexes of proteins to the phosphorylated RTK. Non-receptor tyrosine kinases (NRTKs), such as Src — the first tyrosine kinase to be discovered — are among the recruited proteins. STAT3 and Src can interact independently and STAT3 probably becomes phosphorylated by Src on the EGF and PDGF receptors. Furthermore, it is clear that seven-transmembrane (7TM) receptors can, after binding their peptide or short polypeptide ligands, also activate STAT proteins[152-154]. It has been proposed again that the tyrosine kinase involved is Src — or perhaps the JAKs become activated by associating with 7TM receptors[155-157]. STAT1, STAT3, STAT4, STAT5 and STAT6 homodimerize. STAT1 and STAT2, and STAT1 and STAT3 can form heterodimers, and several STAT proteins can form tetramers (or potentially higher order complexes).

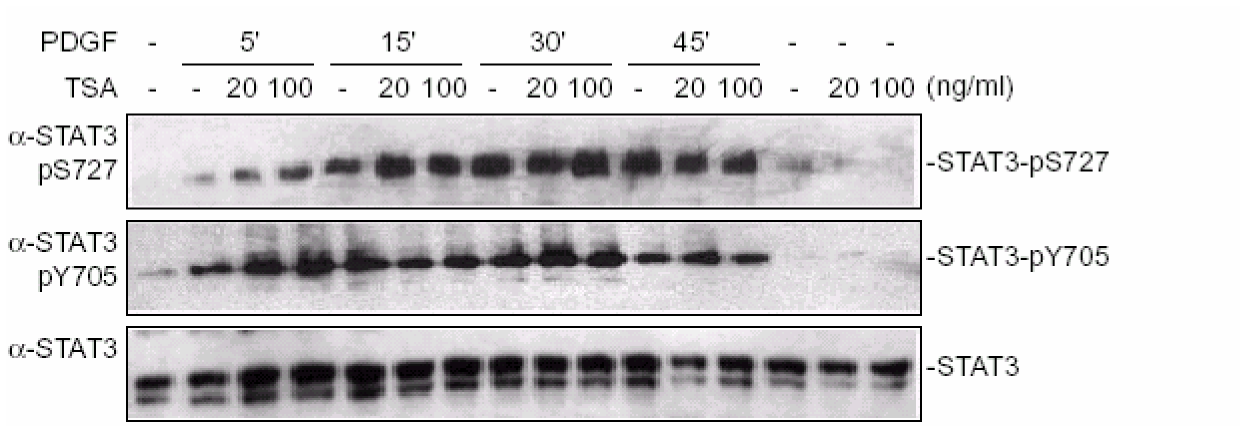

FIG. 9. TSA does not interfere with STAT3 tyrosine[705] and serine[727] phosphorylation upon PDGF stimulation. NIH3T3 cells were starved for 24 hrs, then left untreated or treated with combinations of increasing concentrations of TSA and PDGF, as indicated. Total lysates were assayed by Western blot using specific antibodies: anti-STAT3-[pSer727] (upper panel), anti-STAT3-[pTyr705] (middle panel) and anti-STAT3 (bottom panel). -, no treatment; α-, antibody against.

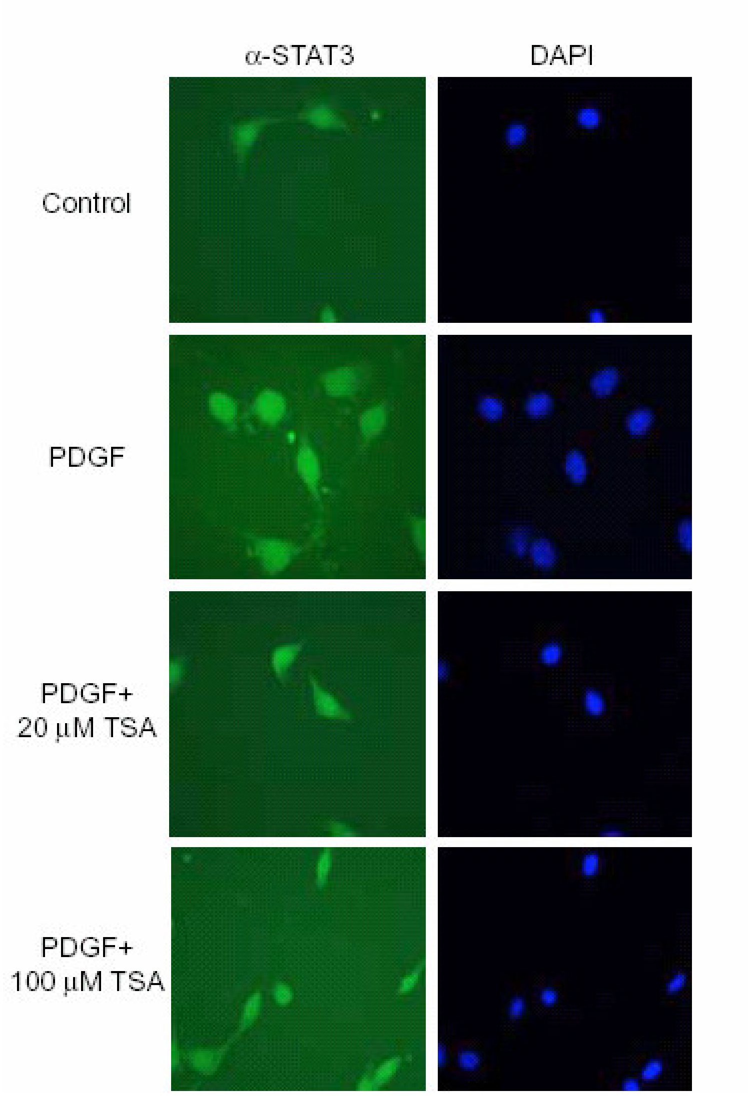

FIG. 10. TSA does not alter the sub-cellular localization of STAT3. NIH3T3 cells were seeded on coverslips and, after 24 hrs, transferred to serum-free medium for an additional 18 hrs. Cells were pretreated with increasing concentrations of TSA and then stimulated with PDGF for 1 hr. Subsequently, cells were fixed and analyzed by immunofluorescence for endogenous STAT3 (a-STAT3) and nuclear staining with DAPI. Comb., combination; a-, antibody against.

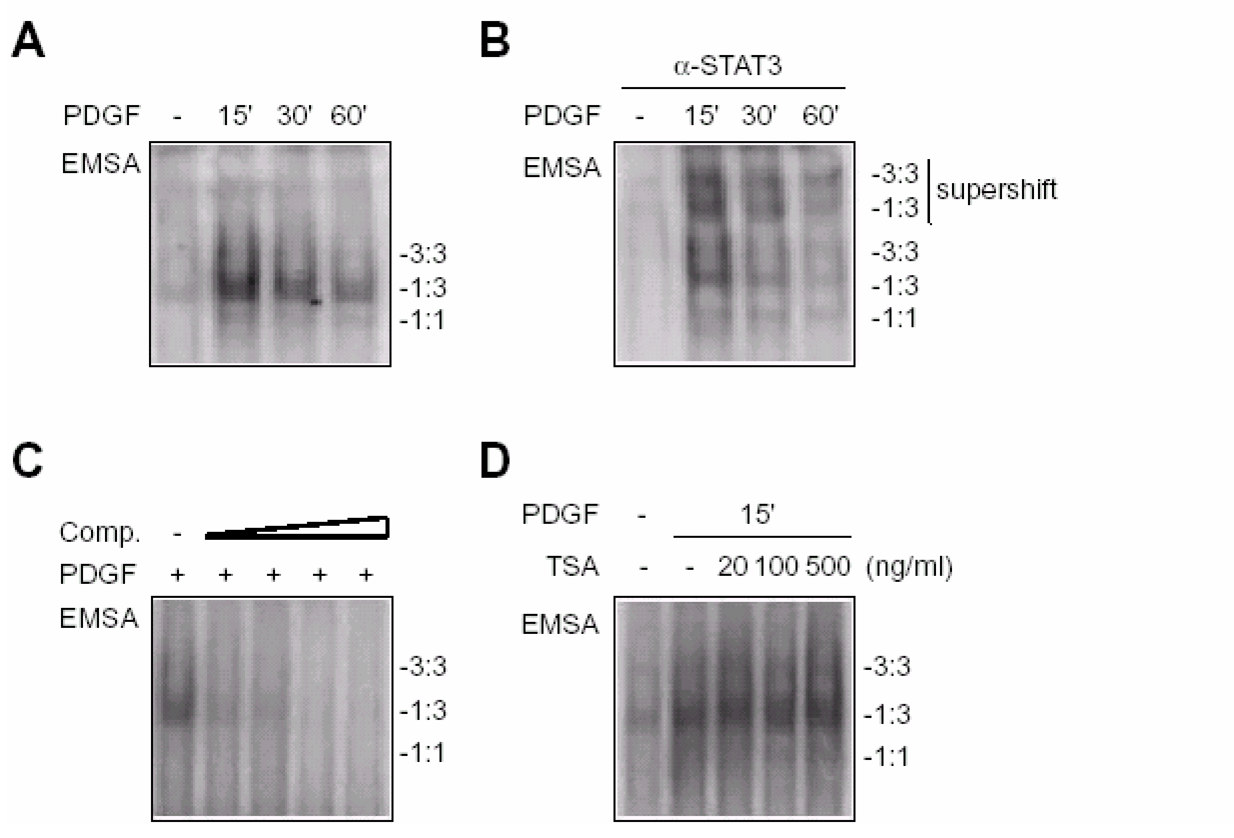

FIG. 11. PDGF-stimulated STAT3 DNA binding activity is independent of HDAC activity. NIH3T3 cells were serum-starved for 24 hrs and then treated as indicated. Nuclear extracts were subjected to elecrophoretic mobility shift assay (EMSA) with a ^{32}P-labeled probe containing a STAT3-responsive element. (A) Nuclear extracts from NIH3T3 cells stimulated with PDGF for the indicated durations. (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 analysis, using the unlabeled oligonucleotides as competitors in concentration five to fifty 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 are indicated. -, no treatment; α-, antibody against; Comp., competitor.

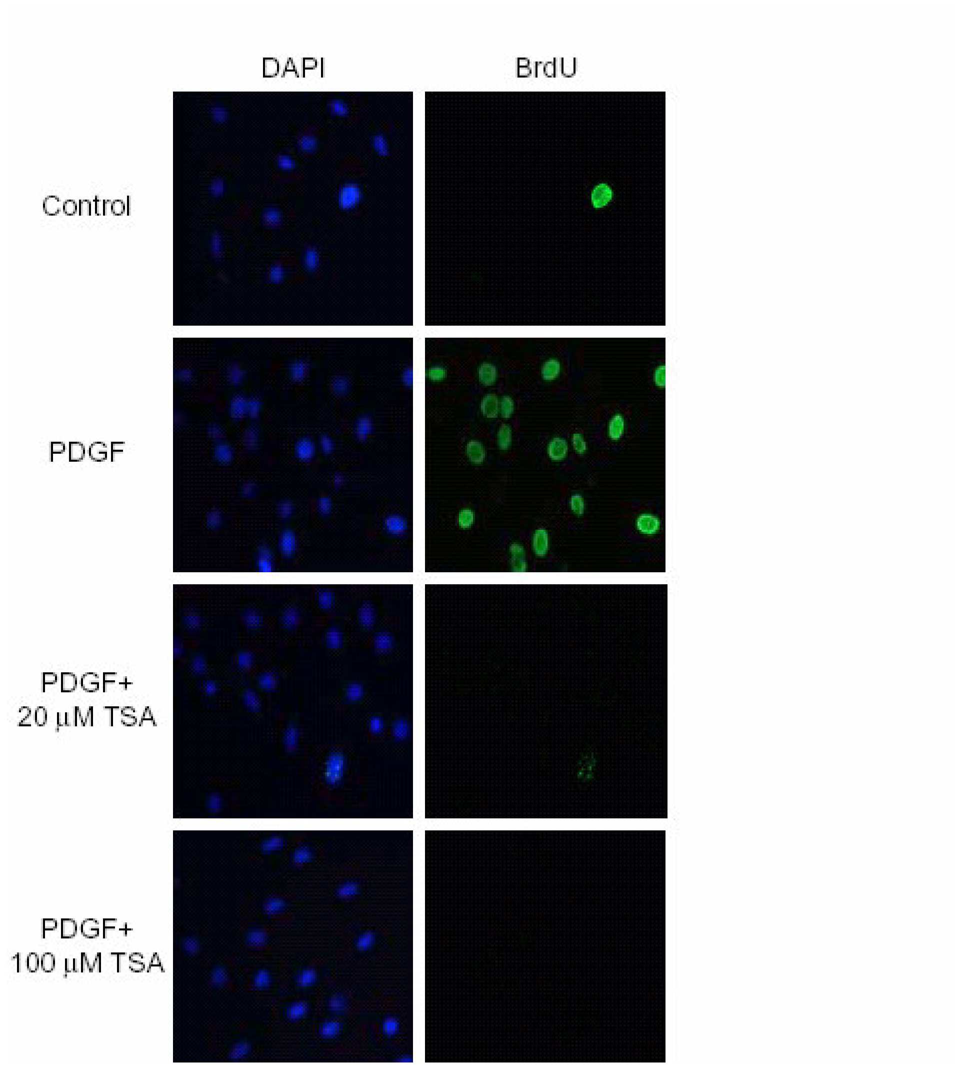

FIG. 12. PDGF-stimulated STAT3 DNA binding activity is independent of HDAC activity. NIH3T3 cells were serum-starved for 24 hrs and then treated as indicated. Nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) with a ^{32}P-labeled probe containing a STAT3-responsive element. (A) Nuclear extracts from NIH3T3 cells stimulated with PDGF for the indicated durations. (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 analysis, using the unlabeled oligonucleotides as competitors in concentration five to fifty 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 are indicated. -, no treatment; α-, antibody against; Comp., competitor.