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ORIGINAL ARTICLE

Basic fibroblast growth factor inhibits p38-mediated cell differentiation and growth inhibition by activin A but not by histone deacetylase inhibitors in CML cells

Chun-Hsin Chen · John Yi-Chung Lin · Fu-Hwa Liu · Ju-Ling Chang · Huei-Mei Huang

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Abstract The p38 mitogen-activated protein kinase (p38) is involved in multiple cellular functions such as cell proliferation and differentiation. Previously, we found that activin A mediated hemoglobin synthesis and cell growth inhibition through p38, whereas, basic fibroblast growth factor (bFGF) inactivated p38 to antagonize the activin A effects. In this study, we selected three structurally different histone deacetylase (HDAC) inhibitors, apicidin, MS275, and sodium butyrate that activate p38, to probe the signal pathway from activin A to p38 in chronic myeloid leukemia (CML)-derived K562 cells. HDAC inhibitors and activin A showed additive p38 phosphorylation. The enhanced phosphorylation of p38 was correlated with increased cell differentiation and decreased cell proliferation. The use of p38 inhibitor SB203580 in conjunction with activin A or with the HDAC inhibitors inhibited cell differentiation and restored cell proliferation, indicating that activin A and the HDAC inhibitors exert their effects through p38 activation. However, bFGF did not affect HDAC inhibitors-induced cell differentiation or growth inhibition. Western blots showed that p38 phosphorylation remained at similar levels with or without bFGF in the presence of HDAC inhibitors. Thus, the HDAC inhibitors activate p38 in a manner different from the activin A pathway. Furthermore, mRNA expressions for activin type I, IB, II, and IIB receptors remained constant in the presence of activin A, bFGF, or both activin A and bFGF. These results indicate that bFGF does not directly act on p38 nor on the mRNA expression levels of activin receptors but inhibit activin A activation of p38 upstream of p38 in K562 cells.

Keywords Activin A · bFGF · Histone deacetylase inhibitor · p38 · Cell proliferation · Cell differentiation

C.-H. Chen
Department of Psychiatry,
Taipei Medical University-Wan Fang Hospital,
Taipei, Taiwan

J. Y.-C. Lin · J.-L. Chang · H.-M. Huang (✉)
Graduate Institute of Cell and Molecular Biology,
Taipei Medical University,
Taipei, Taiwan
e-mail: cmbhhm@tmu.edu.tw

F.-H. Liu
Institute of Molecular Biology, Academia Sinica,
Taipei, Taiwan

Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem/progenitor cell disorder caused by chromosome translocation *t*(9;22), resulting in the formation of the Philadelphia (Ph) chromosome [1, 2]. The Ph chromosome produces an oncogenic Bcr–Abl fusion protein that displays constitutive tyrosine kinase activity [3, 4]. Clinically, CML usually evolves in three stages, an initial chronic phase, an accelerated phase, and a blastic phase (blast crisis). The blastic phase is characterized by aggressive proliferation of immature cells and arrest of differentiation, resulting in the accumulation of leukemic blast cells in blood and bone marrow. The K562 cell line was established from a Ph chromosome-positive CML patient in blastic phase. A number of agents, including chemotherapeutic drugs [5], chemical agents [6–8], and cytokines [9, 10] have been shown to induce the differentiation of K562 cells into different hematopoietic lineages. Therefore, K562 cells provide a useful model to study the biological functions

of CML differentiation using chemical and biological agents.

Activins belong to the transforming growth factor-β (TGF-β) superfamily and was initially isolated in 1986 as a gonadal protein that stimulates the secretion of follicle-stimulating hormone (FSH) from the anterior pituitary cells [11, 12]. In addition to its endocrine function, activins are important in a variety of biological functions including cell proliferation, differentiation, apoptosis, metabolism, homeostasis, and immune responses [13]. Activin A induces erythroid differentiation of hematopoietic progenitor cells both in vitro and in vivo [14, 15]. Activin A signal transduction was shown to be mediated through its type I and type II serine/threonine kinase receptors (further divided into subtypes I and IB, II and IIB) that increased the hemoglobin content of the progenitor cells and limited cell proliferation [15]. Previously, we reported that activin A induced erythroid differentiation by activating p38 mitogen-activated protein kinase (p38) in K562 cells. Furthermore, basic fibroblast growth factor (bFGF) inactivated p38 to antagonize the activin A effects [10]. So far, how bFGF inhibits activin A-induced p38 phosphorylation has not been demonstrated.

Histone deacetylase (HDAC) inhibitors have differentiation, cell-cycle arrest, and apoptotic effects on different cells and are being explored as anticancer drugs. There are many types of HDAC inhibitors that have different chemical and biologic properties but they all can induce the acetylation of histones. Previous studies showed that different structural classes of HDAC-inhibitors (sodium butyrate, apicidin, MS275) exhibited erythroid differentiation inducing activities in K562 cells [16]. Sodium butyrate and apicidin can activate p38 to cause erythroid differentiation [16, 17]. The role of p38 in MS275-mediated erythroid differentiation has not been explored.

There are three aims to this study: (1) to determine the role of p38 in the effects of HDAC inhibitors and activin A, in conjunction with bFGF, on cell growth and differentiation; (2) to decipher the pathway for the suppressive effect of bFGF on activin A differentiation of K562 cells; and (3) to determine if bFGF will antagonize HDAC inhibitors as it does activin A. We show that HDAC inhibitors, which activate p38 and cause erythroid differentiation in K562 cells, are not affected by bFGF. Both activin A and HDAC inhibitors were antagonized by p38 inhibitor SB203580, but bFGF had no effect on any of the HDAC inhibitors, indicating that the additive effects of HDAC inhibitors and activin A have independent activations of p38. Furthermore, the effects of bFGF antagonizing activin A does not alter the gene expression of activin receptors. These results suggest that the inhibitory effect of bFGF on activin A activation of p38 acts downstream of activin receptors and upstream of p38.

Materials and methods

Cell lines and materials

Human K562 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Recombinant activin A was purchased from R&D (Minneapolis, MN). Anti-phospho-p38 and anti-p38 antibodies were purchased from New England Biolabs (Beverly, MA). Sodium butyrate and benzidine were purchased from Sigma Chemical (St. Louis, MO). SB203580, apicidin, and MS275 were purchased from Calbiochem (La Jolla, CA).

Cell proliferation analysis

For analyzing proliferation activity, cells were cultured in growth medium at a starting density of 1×10^5 cells/ml and treated with different agents (100 ng/ml activin A, 100 ng/ml bFGF, 0.1 μM apicidin, 0.1 μM MS275, or 0.3 mM sodium butyrate) for 3 days. The numbers of viable cells were counted by trypan blue exclusion assay at different time points.

Benzidine staining assay

K562 cell cultures at a starting density of 1×10^5 cells/ml were treated with different agents (100 ng/ml activin A, 100 ng/ml bFGF, 0.1 μM apicidin, 0.1 μM MS275, or 0.3 mM sodium butyrate) for 3 days. To detect hemoglobin (Hb) synthesis, cells were suspended in a staining solution of 49:1 ratio of benzidine solution (0.2% benzidine in 0.5% acetic acid) to 30% H_2O_2, and then subjected to cytospin centrifugation after 10 min of incubation at room temperature. The ratios of dark benzidine-stained Hb-positive cells to unstained cells were determined by cytometry.

Western blot analysis

Total cell extracts were prepared as described previously [10]. Protein concentration of samples was determined using Bio-Rad's Bradford protein assay. The phosphorylated proteins were examined by Western blot analysis as described previously [18]. Equal quantities of proteins were electrophoresed on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), then probed with antibodies. After binding with horseradish peroxidase-conjugated secondary antibodies, the blots were visualized with an enhanced chemiluminescence (ECL) detection system (MEN Life Science Products, Boston, MA).

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated from K562 cells as described previously [19]. After purification, 1 μg of RNA was reverse-transcribed at 42°C for 60 min with primer oligo-dT_{18} followed by enzyme inactivation at 70°C for 15 min. The resulting cDNA samples were amplified by polymerase chain reaction (PCR), using the following primers: activin A receptor (ActR)-I sense strand 5′- ATGGTAGATG. GAGTGATG -3′ and antisense strand 5′- AGGAGAA GATCTTCACGG -3′ generated a ~700 bp product; ActR-IB sense strand 5′- ACCTCAAGGAGCCTGAGCAC -3′ and antisense strand 5′- AACGATGGTTCGGGCCACTG -3′ generated a ~270 bp product; ActR-II sense strand 5′- ATGGGAGCTGCTGCAAAG -3′ and antisense strand 5′- TCTTGTGATGCCTGTACA-3′ generated a ~500 bp product; ActR-IIB sense strand 5′- TCCAGGACAAGC AGTCGTGG-3′ and antisense strand 5′-CCAAGCC AAAGTCAGCCAGC-3′ generated a ~346 bp product; β-actin sense strand 5′-GCATCCCCCAAAGTTCACAA- 3′ and antisense strand 5′-AGGACTGGGCCATTCTCCTT-3′ generated a~150 bp product as internal control. The PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light.

Statistical analysis

Data are expressed as the mean ± SE. Intergroup comparisons were performed by one-way analysis of variance (ANOVA) followed by the Dunnett posttest to determine whether there were significant differences among the treatment combinations two at a time. A value of $p<0.05$ is considered statistically significant.

Results

Activin A and HDAC inhibitors acted additively to activate p38 phosphorylation in K562 cells

Activin A has been shown to induce p38 phosphorylation [10]. HDAC inhibitors, apicidin, and sodium butyrate, are also potent inducers of p38 phosphorylation [16, 17]. Whether MS275 activated p38 is not clear. We selected HDAC inhibitors, MS275, apicidin, and sodium butyrate that are chemically diverse but are proposed to act by similar mechanisms against the enzyme HDAC. To determine what effects the HDAC inhibitors have on activin A activation of p38, we compared the phosphorylated signals of p38 by Western blotting. K562 cells were treated with activin A, apicidin, MS275, sodium butyrate, or the combination of activin A plus a HDAC inhibitor. After incubating K562 cells for 3 days with respective agents, the cells were lysed and Western blotting assay was performed. The three HDAC inhibitors, apicidin, MS275, and sodium butyrate, and activin A showed increases in p38 phosphorylation after treatment. Moreover, the concurrent use of both activin A with each HDAC inhibitor caused additive increases in p38 phosphorylation (Fig. 1).

Activin A and HDAC inhibitors acted additively to induce Hb synthesis and to inhibit cell proliferation through p38 activation

To determine the effects of the combination of activin A and HDAC inhibitors on erythroid differentiation and cell proliferation, we cultured K562 cells treated with activin A or each specific HDAC inhibitors, separately and in combinations, for 3 days. Cell differentiation was determined by Hb synthesis in K562 cells using the benzidine staining assay. Treatments with activin A or each specific HDAC inhibitor resulted in increased Hb synthesis vs control. Activin A showed additive effect with each HDAC inhibitors, apicidin, MS275, or sodium butyrate in the Hb synthesis in K562 cells (Fig. 2a). Cell proliferation was determined by trypan blue exclusion assays. Treatments with activin A or each specific HDAC inhibitor resulted in decreased cell proliferation vs control. The combinations of both activin A and each HDAC inhibitor further decreased the number of viable cells after 3 days of incubation (Fig. 2b). Thus, the additive effect of activin A and HDAC inhibitors leads to increased cell differentiation and reduced cell proliferation.

The p38 is additively activated by activin A and each HDAC inhibitor (Fig. 1). To assess the role of p38 activation in the regulation of both cell differentiation and proliferation by both activin A and HDAC inhibitor, we

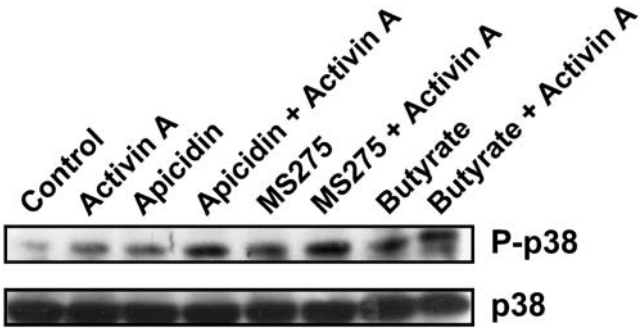

Fig. 1 Activin A and HDAC inhibitors stimulated additive increases in p38 phosphorylation. K562 cells were treated with activin A (100 ng/ml), apicidin (0.1 μM), MS275 (0.1 μM), sodium butyrate (butyrate, 0.3 mM), or a combination of activin A plus a HDAC inhibitor then incubated for 3 days. Prepared cell lysates were subjected to Western blot analysis for the phosphorylation of p38 using specific anti-phospho-p38 (P-p38) antibody, then reprobed with anti-p38 antibody as loading controls. DMSO, used as agent solvent, was included as a negative control treatment (*lane 1*)

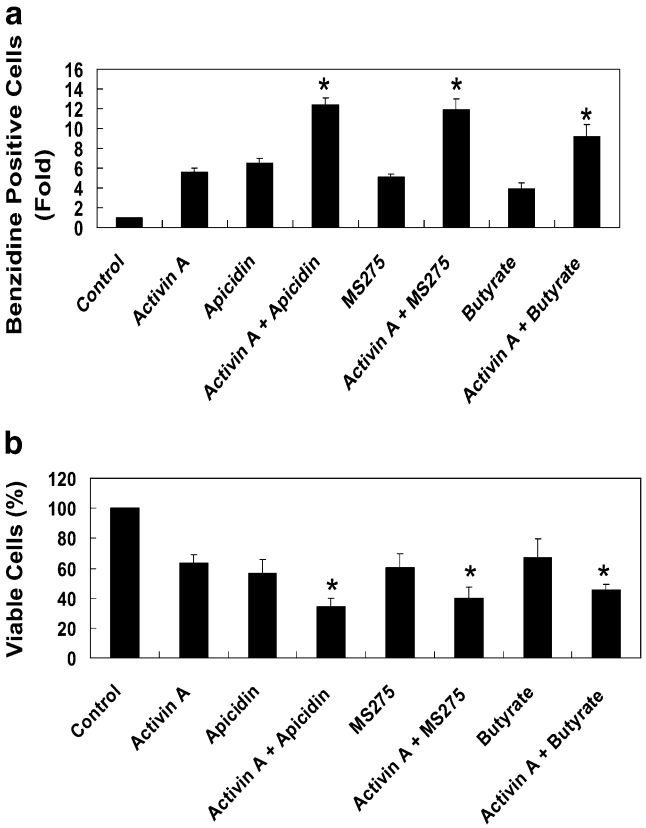

Fig. 2 Activin A and HDAC inhibitors additively increased cell differentiation and decreased cell proliferation. **a** K562 cells were incubated with respective treatments for 3 days, then stained with benzidine solution for Hb synthesis, and counted to determine differentiation activity. Benzidine-positive cells were expressed relative to untreated cells (onefold). **b** Viable cells were determined by trypan blue exclusion assay, cell numbers were normalized to control (100%). The results were determined from four independent experiments and expressed as the average ± SE. Statistically significant differences (*$p<0.05$) were determined by ANOVA followed by the Dunnett posttest for comparisons within treatment combinations, e.g., activin A + apicidin vs activin A alone and vs apicidin alone

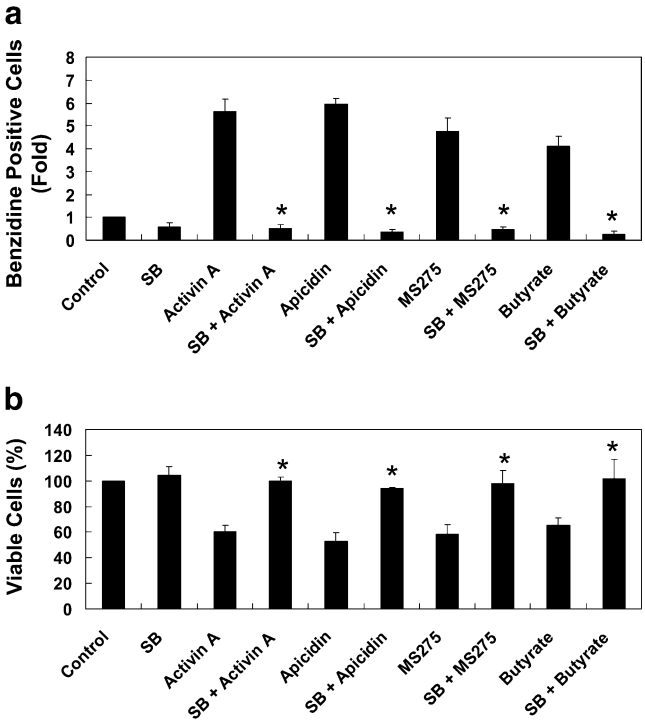

Fig. 3 The p38 inhibitor SB203580 inhibited the potentiating effects of activin A and HDAC inhibitors on cell differentiation and restored the suppressive effects of activin A and HDAC inhibitors on cell proliferation. **a** K562 cells were incubated with respective treatments for 3 days then cells stained with benzidine for Hb positive cells; the benzidine positive cells were expressed relative to untreated cells (onefold). **b** Viable cells were determined by trypan blue exclusion assay and quantitated relative to untreated cells (100%). The results were determined from four independent experiments, shown as the average ± SE. Statistical significances (*$p<0.001$) were determined by ANOVA followed by the Dunnett posttest for comparisons within treatment combinations, e.g., SB + activin A vs activin A alone

treated k562 cells with p38 inhibitor SB203580. SB203580 inhibits the catalytic activity of p38 by competitive binding in the ATP pocket [20]. SB203580 was added at 10 μM, a concentration known to induce selective inhibition of p38 kinase activity in K562 cells [19]. The use of p38 inhibitor blocked activin A- and HDAC inhibitors-mediated K562 differentiation (Fig. 3a). SB203580 also significantly restored activin A and HDAC inhibitor suppression of cell proliferation when compared with cells treated with activin A or each of the HDAC inhibitors (Fig. 3b). These results suggest that activin A and the HDAC inhibitors exert cellular effects through p38 activation.

bFGF inhibits p38-mediated cell differentiation and growth inhibition by activin A but not by HDAC inhibitors

bFGF antagonized activin A-mediated Hb synthesis and cell growth inhibition [10]. We ask if bFGF exert its effect on p38 activation upstream of p38. Thus, we used HDAC inhibitors that show strong activation of p38, induce Hb synthesis, and inhibit cell growth in K562 cells to determine whether bFGF have similar effects on HDAC inhibitors' induction of k562 differentiation and inhibition of cell proliferation. Cells treated with HDAC inhibitors were not dramatically affected by co-incubation with bFGF. The amount of Hb-stained cells were not significantly changed when bFGF was added with HDAC inhibitors compared to HDAC inhibitors alone (Fig. 4a). Although bFGF can inhibit activin A differentiation of K562 cells, it does not have a similar effect on any of the HDAC inhibitors. Similarly, the K562 cell proliferation remained depressed despite the addition of bFGF, in contrast to bFGF effects on activin A-treated K562 cells (Fig. 4b).

bFGF antagonized activin A effects by inactivating p38 [10]. To determine if bFGF can reverse HDAC inhibitor effects by the inhibition of HDAC inhibitor-activated p38, we tested p38 phosphorylation by Western blots. The Western blot shows that the HDAC inhibitors increased p38 phosphorylation just as with activin A, but co-

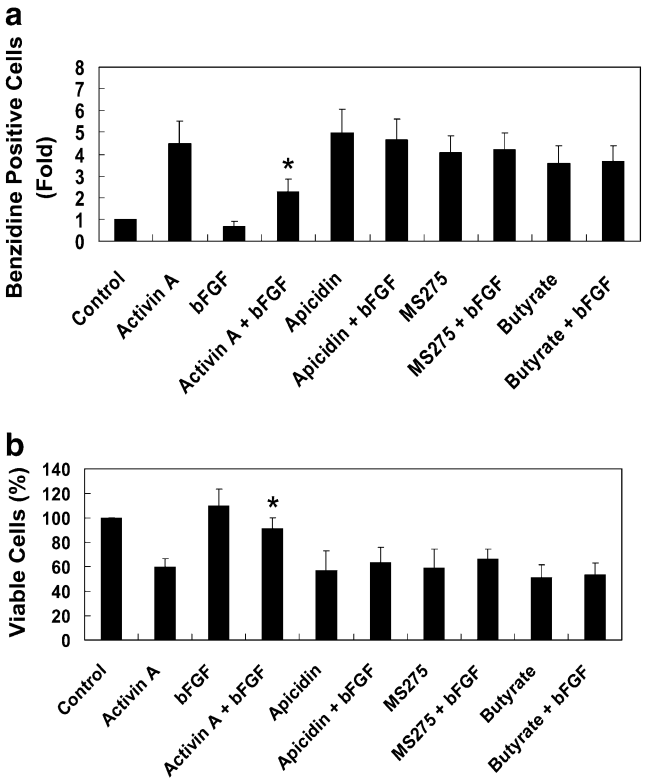

Fig. 4 bFGF inhibited cell differentiation by activin A but not by HDAC inhibitors and reversed activin A inhibition of cell proliferation for activin A but not for HDAC inhibitors. **a** K562 cells were incubated with respective treatments for 3 days, then stained with benzidine solution for Hb synthesis, and counted to determine differentiation activity. Benzidine-positive cells were expressed relative to untreated cells (onefold). **b** Viable cells were determined by trypan blue exclusion assay and quantitated relative to untreated cells (100%). The results were determined from four independent experiments, shown as the average ± SE. Statistical significances (*$p<0.05$) were determined by ANOVA followed by the Dunnett posttest for comparisons within treatment combinations, e.g., activin A + bFGF vs activin A alone

incubation with bFGF showed significant decrease of p38 phosphorylation down to control levels for activin A, but no dramatic changes in p38 activation for any of the HDAC inhibitors. p38 phosphorylation did not decrease significantly in the co-treatment of HDAC inhibitors with bFGF (Fig. 5).

Analysis of expression of the activin A receptor genes by activin A and bFGF

To explore if bFGF inhibit activin A effects by down-regulating the expression of activin A receptor genes in K562 cells, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. K562 cells were exposed to activin A, bFGF, or to both activin A and bFGF for 3 days or left untreated (Fig. 6). Total RNA was isolated from four independent experiments and processed for RT-PCR. These data indicate that activin A receptor (ActR) isoforms, type I

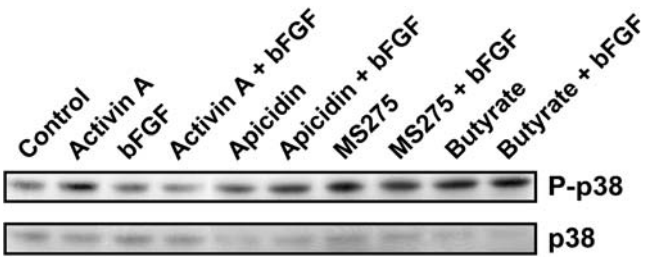

Fig. 5 bFGF decreased p38 phosphorylation due to activin A, but did not affect p38 phospohorylation due to HDAC inhibitors. K562 cells were treated with activin A (100 ng/ml), bFGF (100 ng/ml), apicidin (0.1 μM), MS275 (0.1 μM), sodium butyrate (butyrate, 0.3 mM), the combination of bFGF plus activin A, or the combination of bFGF plus each HDAC inhibitor, then incubated for 3 days. Cell lysates were prepared and subjected to Western blot analysis for the phosphorylation of p38 using specific anti-phospho-p38 (P-p38) antibody, then reprobed with the anti-p38 antibody as loading controls. DMSO was used as solvent agent; therefore, DMSO treatment was included as a negative control treatment (*lane 1*)

(ActR-I and ActR-IB) and type II (ActR-II and ActR-IIB) receptors, were not altered by treatment with activin A and bFGF.

Discussion

Hematopoiesis is a complicated process from which a small population of stem cells develops into large populations of mature blood cells. These diverse proliferation, differenti-

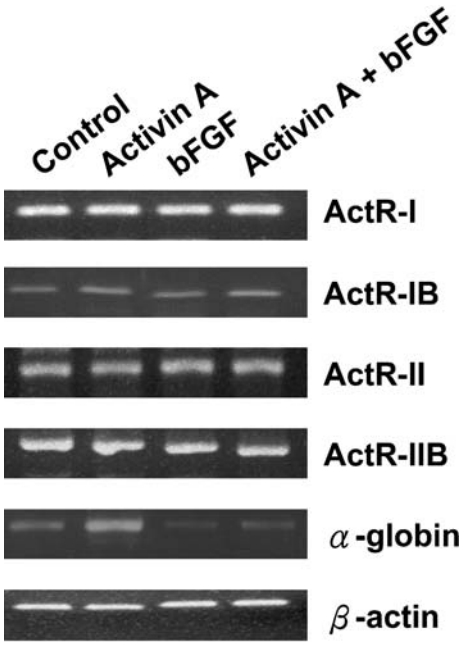

Fig. 6 Gene expressions of activin A receptors not affected by activin A and bFGF treatments. K562 cells were treated with or without (control) 100 ng/ml activin A, 100 ng/ml bFGF, or a combination of both for 3 days. RNA isolated from these distinct cultured cells were submitted to RT-PCR analysis as described in the Materials and methods section, followed by electrophoresis on 2% agarose gel

ation, and maturation events are achieved by a network of multiple hematopoietic cytokines. It is known that proliferation cytokines and differentiation cytokines are important factors in regulating cell fate of hematopoietic progenitors; hence, it is critical to characterize their intracellular signaling mechanisms. K562 is a primitive type mylogenic cell line that can differentiate into myeloid or erythroid fates [16, 21, 22]. Our earlier study showed that stimulation by activin A (a differentiation cytokine) decreased cell proliferation and increased erythroid differentiation associated with p38 activation [10]. bFGF (a proliferation cytokine) antagonized activin A to promote cell proliferation and inhibit cell differentiation accompanied by the inhibition of p38 activation. The site of action for bFGF on the signal pathway between activin A and p38 remain undefined.

In the current study, we show that HDAC inhibitors, apicidin, MS275, or sodium butyrate, additively enhanced the effect of activin A on the erythroid differentiation and growth inhibition of K562 cells. It has been reported that apicidin and sodium butyrate induced erythroid differentiation by activating p38 [16, 17]. Our present results show that MS275 could activate p38 to induce erythroid differentiation. Several studies have reported that HDAC inhibitors play a role in the developmental control of hemoglobin gene expression [16, 23–25] as discussed previously [16]. We also provide evidence that p38 was the essential factor not only for inducing erythroid differentiation, as reported [26, 27], and for inhibiting cell proliferation in HDAC inhibitors-mediated and activin A-mediated effects. However, HDAC inhibitors, which induced erythroid differentiation in K562 cells and activated p38 were not significantly affected by bFGF. The enhanced effect of HDAC inhibitors and activin A on p38 activation indicated that both agents may independently activate p38 through different signaling pathways. bFGF inhibition of p38 activation by activin A is not directed specifically at p38 but acts upstream of p38 (Fig. 7). Both activin A and the HDAC inhibitors were antagonized by p38 inhibitor SB203580, but bFGF have no effect on any of the HDAC inhibitors.

For activin A signaling, ligand binding to either of its known activin type II receptors (ActR-II or ActR-IIB) results in the recruitment of and trans-phosphorylation of activin type I receptors (ActR-I or ActR-IB); the activated type I receptors mediate signaling to downstream targets [15, 28–30]. All four known activin receptor subtypes (ActR-I, ActR-IB, ActR-II, and ActR-IIB) are expressed in K562 cells [31, 32]. It has been reported that activin type I and IB receptor mRNA levels are induced during TPA-induced differentiation of K562 [32]. As the inhibitory effect of bFGF is upstream of p38 activation by activin A (Fig. 7), one possibility is that bFGF influences and

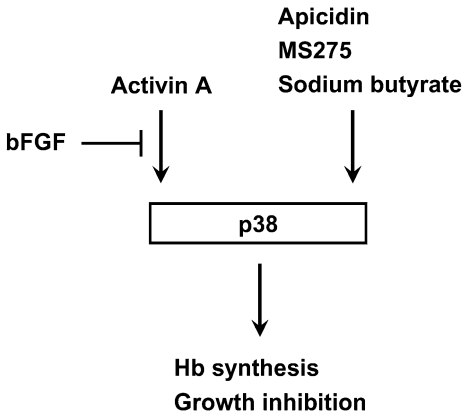

Fig. 7 Schematics of p38 activation by activin A and HDAC inhibitors. bFGF was shown to counteract activin A effects, while not affecting the HDAC inhibitors. The additive activation of p38 suggests that activin A and the HDAC inhibitors independently activate p38 through different pathways. p38 activation in K562 cells lead to increased erythroid differentiation and inhibition of cell proliferation

interacts with elements close to the membrane activin A receptors. Our present results indicate that the mRNA levels of the four activin receptor subtypes are not regulated by bFGF or activin A. These results are consistent with the previous report in which no changes in activin type I and II receptor mRNA levels were detected upon differentiation of K562 cells by activin A [31, 32].

As bFGF does not down-regulate the expression level of activin A receptors to cause the decrease in p38 phosphorylation and activin A-mediated differentiation, we can speculate on other possible ways that bFGF can inhibit activin A-mediated p38 activation. First, bFGF can act on signaling elements upstream of p38, i.e., on p38 activators (MKK3/MKK6 and TAB1), to inhibit p38 phosphorylation. MKK3 (MEK3) and MKK6 (MEK6) show a high degree of specificity for p38 activation, as they do not activate ERK1/2 (extracellular regulated kinase) or JNK (jun N-terminal kinase) [33, 34]. The ability of MKK3/MKK6 to directly activate globin genes was confirmed by studies using constitutively activated p38 in K562 stable cell lines [25]. The protein TAK1 (TGF-β activating kinase) and its interacting protein (TAB1) can be activated by TGF-β1, an analog of activin A. Furthermore, TAB1 have been shown to provide an alternative pathway to p38 activation and to specifically activate p38 [35]. Second, an important feature in the inverse relationship between p38 and ERK activities in the regulation of keratinocyte differentiation was reported: p38 directly interacted with ERK to inhibit ERK activity [36]. Our previous results have shown that bFGF activated ERK to antagonize activin A-mediated erythroid differentiation in K562 cells and also deactivated the p38 pathways [10]. It is not clear whether bFGF-mediated ERK activation and p38 inactivation in K562 cells are by direct interactions between ERK and p38. Third,

another possible way for bFGF to regulate activin A activation of p38 is through negative regulators, such as MAPK phosphatases (MPK-7), which dephosphorylates activated p38. The increased dephosphorylation of activated p38 would counteract activin A phosphorylation of p38. MPK-7, a specific p38 phosphatase, binds to and inactivated p38 and JNK but not ERK [37].

Thus, we are working on multiple tracks in our laboratory to clarify the point of interaction between bFGF and activin A pathways and its influence on p38 pathway in the regulation of erythroid differentiation and cell proliferation. The difference between activin A and HDAC inhibitor for p38 activation is also an interesting point to resolve.

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