

Regulation of Id1 Expression by Src: Implications for Targeting of the Bone Morphogenetic Protein Pathway in Cancer

Oliver Gautschi,^{1,2} Clifford G. Tepper,^{2,3} Phillip R. Purnell,² Yoshihiro Izumiya,³ Christopher P. Evans,⁴ Tim P. Green,⁵ Pierre Y. Desprez,⁶ Primo N. Lara,² David R. Gandara,² Philip C. Mack,² and Hsing-Jien Kung^{2,3}

¹Department of Medical Oncology, Bern University Hospital, Bern, Switzerland; ²University of California Davis Cancer Center, Departments of ³Biochemistry and Molecular Medicine, and ⁴Urology, School of Medicine, University of California Davis, Sacramento, California; ⁵AstraZeneca Pharmaceuticals, Alderley Park, United Kingdom; and ⁶California Pacific Medical Center, Cancer Research Institute, San Francisco, California

Abstract

Deregulated activation of the Src tyrosine kinase and heightened Id1 expression are independent mediators of aggressive tumor biology. The present report implicates Src signaling as a critical regulator of Id1 gene expression. Microarray analyses showed that Id family genes were among the most highly down-regulated by incubation of A549 lung carcinoma cells with the small-molecule Src inhibitor AZD0530. Id1 transcript and protein levels were potently reduced in a dose-dependent manner concomitantly with the reduction of activated Src levels. These effects were conserved across a panel of lung, breast, prostate, and colon cancer cell lines and confirmed by the ability of PP2, Src siRNA, and Src-blocking peptides to suppress Id1 expression. PP2, AZD0530, and dominant-negative Src abrogated Id1 promoter activity, which was induced by constitutively active Src. The Src-responsive region of the Id1 promoter was mapped to a region 1,199 to 1,360 bps upstream of the translation start site and contained a Smad-binding element. Src was also required for bone morphogenetic protein-2 (BMP-2)-induced Id1 expression and promoter activity, was moderately activated by BMP-2, and complexed with Smad1/5. Conversely, Src inhibitors blocked Smad1/5 nuclear translocation and binding to the Src-responsive region of the Id1 promoter. Consistent with a role for Src and Id1 in cancer cell invasion, Src inhibitors and Id1 siRNA decreased cancer cell invasion, which was increased by Id1 overexpression. Taken together, these results reveal that Src positively interacts with the BMP-Smad-Id pathway and provide new ways for targeted inhibition of Id1. [Cancer Res 2008;68(7):2250–8]

Introduction

The Src family of nonreceptor protein tyrosine kinases contains nine members, including Src, Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk, and Yrk (1). Src is activated by growth factor receptors, cytokine receptors, protein tyrosine phosphatase 1B, CAS, and focal adhesion kinase (FAK). Src interacts with a network of intracellular signaling pathways, including the integrin/FAK pathway, β -catenin/Wnt, RAS-MEK, phosphatidylinositol-3-OH kinase-AKT

and Janus-activated kinase-STAT pathways. These complex interactions explain why Src is involved in a large number of cellular functions including adhesion, migration, invasion, survival, proliferation, differentiation, inflammation, and angiogenesis. Activated Src induced transformation in fibroblasts, and Src kinases were found frequently to be overexpressed and activated in human cancer (2). This prompted the development of a number of small-molecule Src kinase inhibitors that reduced cancer invasion and metastasis in preclinical models. For example, AZD0530, a potent and selective small-molecule inhibitor of Src kinase, is currently being tested in phase II clinical trials in patients with cancer (3, 4). Apart from their promising clinical utility, small-molecule inhibitors of Src possess the potential to identify genes regulated by Src signaling and putative effector molecules.

The inhibitor of DNA binding/differentiation (Id) family of helix-loop-helix (HLH) proteins comprises four members (Id1-4) that all lack a DNA-binding domain (5). Id proteins associate with and inhibit the function of basic HLH transcription factors, including MyoD and E-proteins, to regulate normal cell fate determination, differentiation, and angiogenesis (6–9). Expression of Id1 is induced by bone morphogenetic proteins (BMP), which activate Smad1/5 via the BMP-receptors (10–13). Activated Smad1/5 binds Smad4, translocates to the nucleus, binds to Smad-binding elements in the Id1 promoter, and recruits transcription factors and coactivators that induce Id1 transcription (14–16). In contrast, transforming growth factor (TGF) β can repress the Id1 promoter by activation of Smad3 (17). BMPs and Id1 are overexpressed in various cancer types and are associated with an aggressive, invasive phenotype (18–22). Expression of Id gene family expression is mediated by oncogenic RAS, MYC, and TP53 gain of function mutation (12, 23–25). Id1 promotes invasion by production of a 120-kDa gelatinase, mediates tumor angiogenesis by production of vascular endothelial growth factor, facilitates hormone-independent growth, and is involved in the resistance of cancer cells against cytotoxic drugs (19, 26, 27). Due to its role in cell differentiation and in vascular endothelial cells, Id1 has also been implicated in the biology of cancer stem cells and tumor angiogenesis (8, 28, 29). Altogether, there is strong evidence that Id1 is an interesting drug target in cancer (30). However, strategies of Id targeting have thus far been limited to methods of gene silencing in the laboratory. Thus, the availability of pharmaceutical methods to inhibit Id1 *in vivo* may greatly advance the understanding of the role of Id1 in the biology, therapy, and prevention of cancer.

Here, we show that Src interacts with and is a positive modulator of the BMP-2/Smad1/Id1 signaling pathway in lung cancer cells, suggesting an important role for Id1 in Src-mediated invasion. We also provide evidence that Src inhibition by small

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Oliver Gautschi, Department of Medical Oncology, Bern University Hospital, 3010 Bern, Switzerland. Phone: 41-31-632-41-14; Fax: 41-31-632-41-20; E-mail: oliver.gautschi@insel.ch.

©2008 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-07-6403

molecules significantly reduces the level of Id1 in lung, breast, prostate, and colon cancer cells, thereby providing a potential way to target Id1 *in vivo*.

Materials and Methods

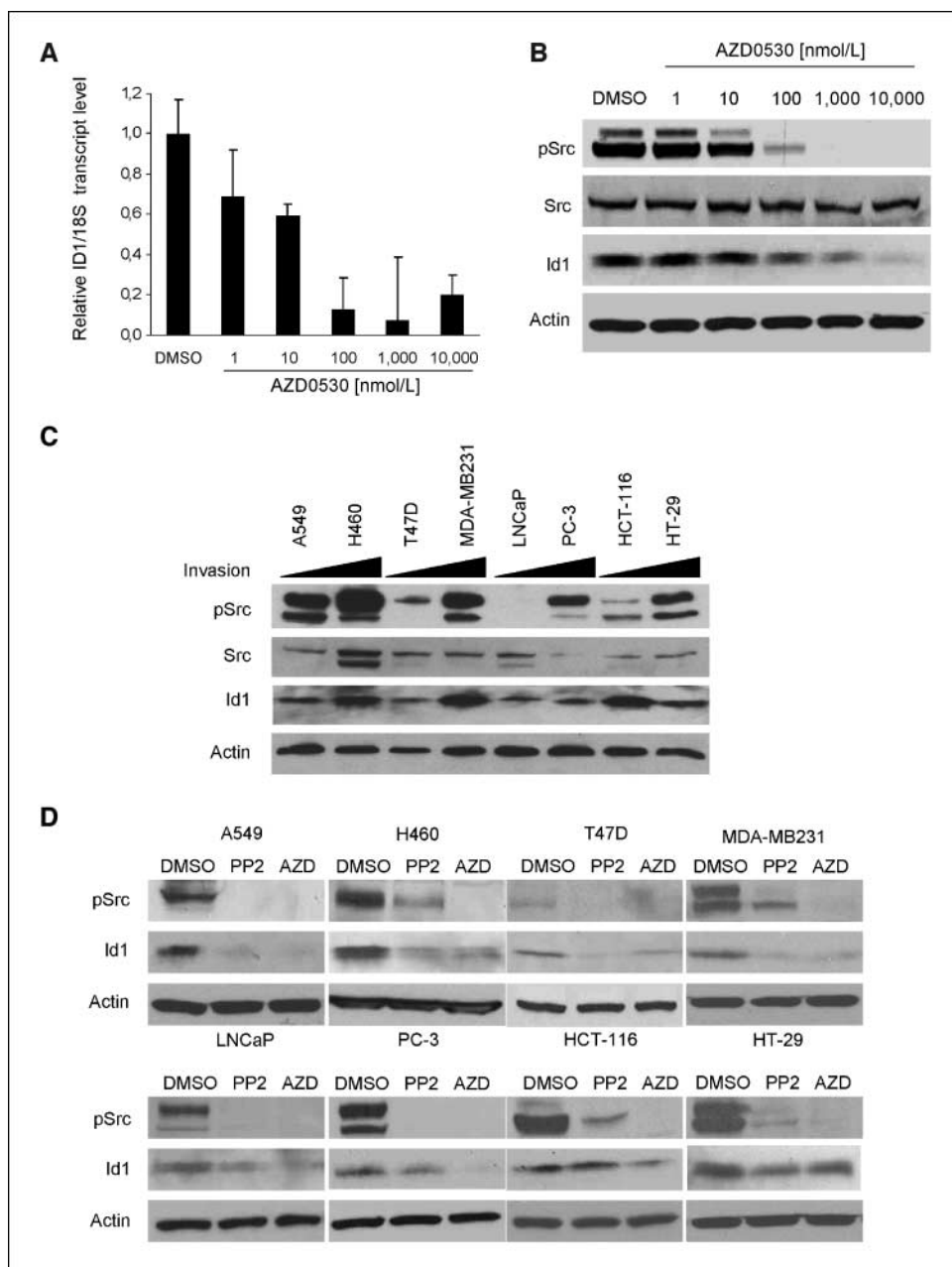
Cell lines and reagents. The following cell lines were obtained from the American Type Culture Collection: A549, H460, LNCaP, PC-3, T47D, MDA-MB231, HCT-116, and HT-29. Cells were cultured in RPMI (LNCaP and PC-3) or DMEM (all other cell lines) plus 10% filtered, heat-inactivated fetal bovine serum (FBS). PP2 (Calbiochem) and AZD0530 (AstraZeneca) were solubilized in DMSO to obtain a 10 and 1 mmol/L stock solution, respectively. The Src-blocking peptides CpraYKYY- β Ala-r7 and CpraYKYY- β Ala-k7 (provided by Dr. Kit Lam, University of California Davis Cancer Center, Sacramento, CA) were solubilized in sterile H₂O to obtain a 25 mmol/L stock solution (31). Recombinant human BMP-2 (R&D Systems) was reconstituted in 4 nmol/L HCl containing 0.1% bovine serum albumin (BSA)

to obtain a 10 μ g/mL stock solution. Stock solutions were stored at -20°C and diluted in DMEM for each experiment.

Microarray gene expression profiling. RNA isolation, RNA purification, and genome-wide expression profiling using Human Genome U133 Plus 2.0 GeneChip arrays (Affymetrix) was performed according to the manufacturer's protocols and as described previously (24). Initial data processing (e.g., signal detection and scaling) for each chip was performed using Affymetrix GeneChip Operating Software. Model-based expression analysis (using the perfect match-mismatch model) was used to identify differentially expressed genes using DNA-Chip Analyzer software (dChip; ref. 32). For this, signals from all of the arrays were normalized to the array that had the median overall intensity. Criteria for the selection of genes exhibiting significant expression changes included an average fold change of ≥ 2.0 (AZD0530/DMSO), P values of ≤ 0.05 , and at least 100 units of change between the two treatments being tested.

Reverse transcription-PCR. Total RNA was extracted using the Trizol protocol (Invitrogen) and cDNA was generated using M-MuLV reverse

Figure 1. Id gene expression is down-regulated by Src inhibition in cancer cells. A549 cells were incubated with increasing concentrations of AZD0530, and control cells were incubated with DMSO. *A*, RNA was isolated after 24 h, and real-time RT-PCR for Id1 RNA and 18S rRNA was performed in triplicates using Sybr Green. Id1 levels were normalized for 18S rRNA. *Columns*, mean relative to control; *bars*, SEs. *B*, protein was isolated after 48 h of incubation with AZD0530, and Western blotting was performed to determine the levels of pY419-Src, Src, Id1, and actin. *C*, lung (A549 and H460), breast (T47D and MDA-MB231), prostate (LNCaP and PC-3), and colon (HCT-116 and HT-29) cancer cells were selected according to their reported invasive potential, and Western blotting was performed to compare the basal levels of pY419-Src, Src, Id1, and actin. *D*, cells were incubated for 48 h with PP2 (10 μ mol/L) or AZD0530 (1 μ mol/L; AZD). Control cells were incubated with DMSO. Western blotting was performed to determine the levels of pY419-Src, Id1, and actin.



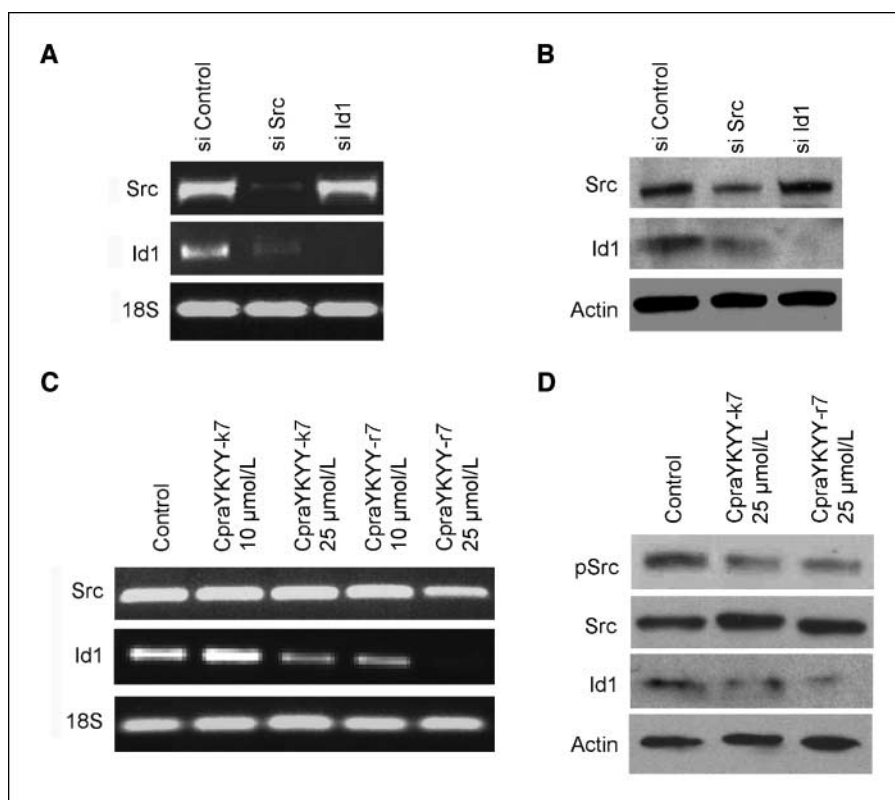


Figure 2. Reduction of Id1 mRNA and protein levels by Src siRNA and blocking peptides. **A**, A549 cells were incubated with Src siRNA, Id1 siRNA, or nontargeting control siRNA (each at 100 nmol/L; 24 h). RNA was isolated and RT-PCR was performed using specific primers for Src mRNA, Id1 mRNA, and 18S rRNA. **B**, protein was isolated for Western blotting and probing for Src, Id1, and actin. **C**, A549 cells were incubated with the Src-blocking peptides CpraYKYY- β Ala-k7 and CpraYKYY- β Ala-r7 at the concentrations indicated for 24 h. RNA was isolated and RT-PCR was performed using specific primers for Src mRNA, Id1 mRNA, and 18S rRNA. **D**, Western blotting was performed to determine the levels of pY419-Src, Src, Id1, and actin.

transcriptase (Fermentas). For PCR primer sequences and annealing temperatures, see Supplementary Fig. S2. PCR products were loaded onto 1% agarose gels and run at 110 V for 1 h.

Real-time RT-PCR. Quantitative real-time PCR was performed using iQ Sybr Green Supermix and the iCycler detection system (Bio-Rad). Primer sequences were as follows: ID1 forward, 5'-CTCTACGACATGAACGGCTGT-3'; ID1 reverse, 5'-TGCTCACCTTGCGGTTCTG-3'; 18S forward, 5'-CGCCGCT-AGAGGTGAAATTCT-3'; and 18S reverse, 5'-CGAACCTCCGACTTTCGTTCT-3'. Standard dilutions, melting curve analysis, and agarose gel electrophoresis of PCR products were performed to confirm accuracy. Triplicate Id1 expression values were normalized for 18S rRNA, and data were processed using Q-GENE software (33).

Western blotting. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer on ice for 30 min and protein concentrations were determined using the bicinchoninic acid protein assay (Pierce Biotechnology). Equal amounts of protein were electrophoresed on polyacrylamide gradient gels (4–20%) and transferred onto nitrocellulose membranes by semidry blotting. Membranes were blocked for 1 h with TBS containing 5% nonfat dry milk and incubated overnight with antibodies against Src (Upstate), phospho-Src family (Cell Signaling), Id1 (Biocheck), pS463/465-Smad1/5 (Upstate), Smad1/5/8 (Santa Cruz Biotechnology), and β -Actin (Santa Cruz Biotechnology). Membranes were washed in TBS and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (Promega). Visualization was performed using enhanced chemiluminescent (ECL) detection reagent and ECL X-ray films (Amersham).

Immunoprecipitation. Cells were lysed with RIPA buffer as described above. Equal amounts of protein were processed using the ExactaCruz kit (Santa Cruz Biotechnology) following the manufacturer's protocol. Briefly, anti-Src antibody (Upstate) was mixed with immunoprecipitation matrix and incubated overnight at 4°C. The mix was centrifuged, and cell lysates were added and incubated overnight at 4°C on a rotator. After centrifugation, pellets were washed in PBS, resuspended in Laemmli buffer, and boiled at 95°C for 5 min. Samples were subjected to SDS-PAGE and Western blotting for Smad1/5/8 (Santa Cruz Biotechnology) and Src (Upstate).

RNA interference. Standard siCONTROL (D-001210-02), on-target plus SMART pool human Src (L-003175-00), and on-target plus SMART pool human Id1 (L-005051-00) were purchased (Dharmacon). Oligonucleotides were complexed with Lipofectamine in Opti-MEM (Invitrogen) according to the manufacturer's protocol and delivered to cells at a final concentration of 100 nmol/L.

Id1 promoter assays. A549 cells were transfected in 96-well plates for 24 h with previously described ID1pGL-luc reporter and SV40pRL coreporter plasmids at a ratio of 10:1 in the presence of Effectene (Qiagen) in 10% FBS DMEM (34). For Src inhibitor assays, cells were then incubated for 24 h with DMSO, PP2, or AZD0530 in 10% FBS DMEM. Samples were lysed and analyzed using the Dual-Luciferase Reporter Assay system (Promega) on a MicroLumat luminometer (EG & G Berthold). For Src mutant assays, cells were triple transfected for 24 h with ID1pGL, SV40pRL plus PCI vectors containing wild-type human Src, dominant-negative human SrcK298M (provided by Dr. Don Fujita, University of Calgary, Alberta, Canada), or constitutively active chicken SrcY527F (provided by Dr. June Zhou, University of California Davis Cancer Center, Sacramento, CA; ref. 35). Cells were then incubated in fresh 10% FBS DMEM for 18 h followed by serum starvation in DMEM for 6 h. For promoter region assays, cells were triple-transfected for 24 h with Src-Y527F, SV40pRL, plus full-length ID1pGL or one of seven previously described ID1 promoter 5' deletion constructs (34). Cells were then incubated in fresh 10% FBS DMEM for 18 h followed by serum starvation in DMEM for 6 h. Assays were performed in triplicates, firefly luciferase activity was normalized for *Renilla* luciferase activity, and relative Id1 promoter activity was calculated based on the mean value of the respective control.

Chromatin immunoprecipitation. A549 cells were incubated with DMSO, PP2, or AZD0530 for 23 h followed by stimulation with BMP-2 (10 ng/mL; 1 h). Cells were cross-linked with 1% formaldehyde for 10 min and incubated in 0.125 mol/L glycine for 5 min. Plates were scraped and cells were centrifuged. Pellets were resuspended in swelling buffer containing 100 mmol/L Tris, 10 mmol/L KOAc, 15 mmol/L MgOAc, and protease-inhibitor cocktail (Roche); incubated for 20 min on ice; and dounce homogenized 15 times. Nuclei were centrifuged; resuspended in

buffer containing 10 mmol/L EDTA, 50 mmol/L Tris-HCl, 0.5% SDS, and protease inhibitor cocktail; and sonicated using a BioRuptor (Diagenode). Lysates were diluted in buffer containing 150 mmol/L NaCl, 2 mmol/L EDTA, 20 mmol/L Tris-HCl, 1% Triton-X, and protease inhibitor cocktail, and split into two aliquots for overnight incubation with 5 μ g of anti-Smad1/5/8 antibody (Santa Cruz Biotechnology) or rabbit IgG (Oncogene Science). Samples were then incubated with 5 μ g of sonicated salmon sperm DNA (Sigma-Aldrich) and 50 μ L of protein G agarose (Upstate) for 2 h. After centrifugation, supernatant was stored (input control) and pellets were washed in TSE buffer [1% TritonX-100, 0.1% SDS, 2 mmol/L EDTA, and 20 mmol/L Tris-HCl (pH 8.1)], eluted in TE buffer (10 mmol/L Tris-HCl and 1 mmol/L EDTA), and incubated at 65°C overnight to reverse the cross-linking. Samples were incubated with 1 μ L of Proteinase K (Fermentas) for 1 h at 55°C, and DNA was isolated using the QIAquick PCR purification kit (Qiagen). PCR for the *Src*-responsive region was performed using forward primer 5'-AATTGTTGGGATTACAGCGGTG-3' and reverse primer 5'-CTGGGAATGCGTTTCTTTCG-3' at an annealing temperature of 55°C for 35 cycles. PCR products were separated on 1.5% agarose gels.

Immunofluorescence. A549 cells in chamber slides were incubated with DMSO, PP2, or AZD0530 for 23 h followed by stimulation with BMP-2 (10 ng/mL; 1 h). Cells were then fixed with 3.7% formaldehyde, permeabilized with Triton-100, blocked with 0.5% BSA in PBS, and incubated with anti-Smad1/5/8 antibody (Santa Cruz Biotechnology) overnight. Cells were washed and incubated with Alexa-647-conjugated

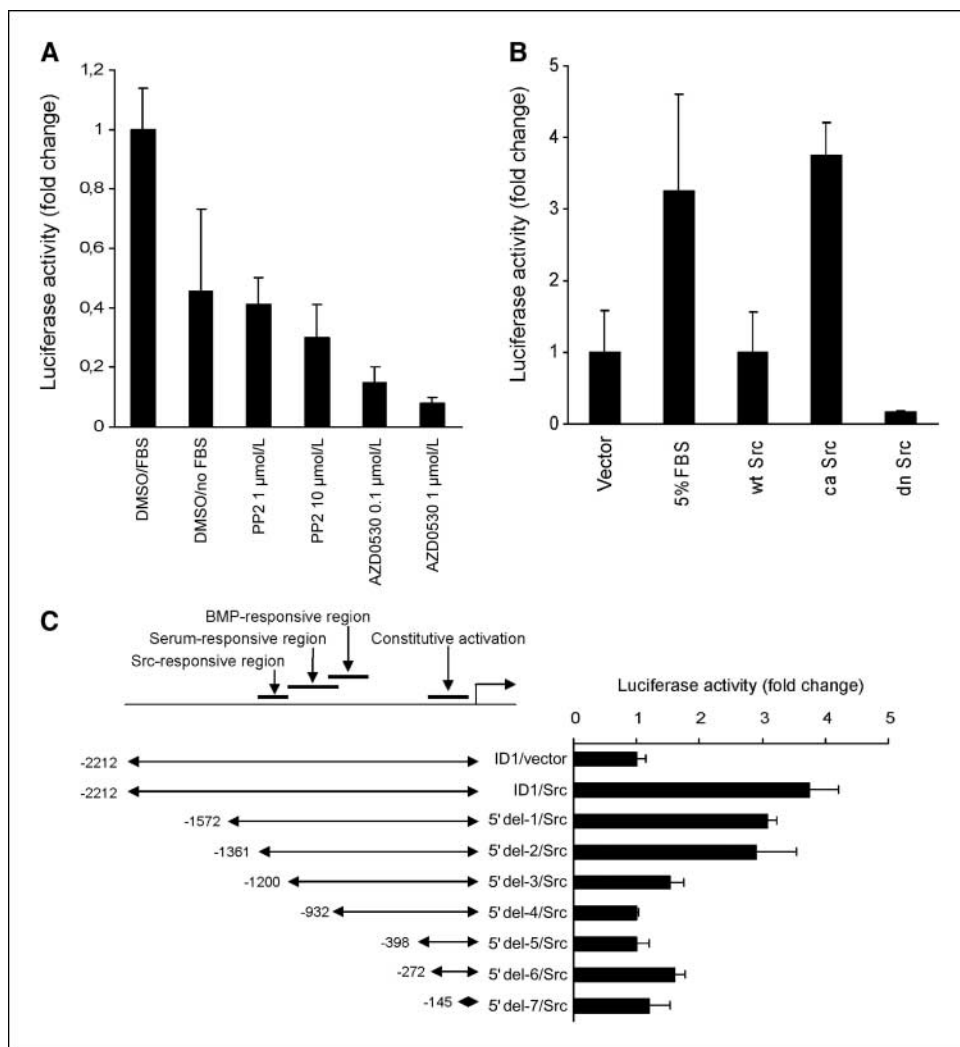
secondary antibody and Hoechst dye. After washing, slides were analyzed using a BX61 microscope and SlideBook 4.1 imaging software (Olympus).

Establishment of a stable *Id1*-overexpressing A549 subline. The full-length *Id1* cDNA sequence was subcloned from pBabe-*Id1* (26) into pLNCX2 retroviral vector (BD Clontech) for cytomegalovirus promoter-driven expression. For production of retrovirus, pLNCX2-*Id1* and pLNCX2-*empty* expression constructs (2 μ g) were transfected into LinX-A amphotropic packaging cells using FuGENE 6 transfection reagent. Cultures were incubated at 32°C. After 72 h, virus-containing supernatant was collected, centrifuged at 3,000 \times *g* for 15 min at 4°C, and filtered through a 0.45- μ m surfactant-free cellulose acetate membrane (Corning, Inc.). A549 cells were then infected with a mix of DMEM, virus-containing supernatant (1:1), and polybrene (4 μ g/mL). After incubation for 24 h at 32°C, cells were selected in 10% FBS DMEM with 400 μ g/mL of Geneticin (JR Scientific) at 37°C for 3 wk.

Invasion assays. Cell culture inserts with polyethylene terephthalate membranes and 8- μ m pores were coated with 60 μ L Matrigel (BD Biosciences). Top chambers were filled with A549 cells in 5% FBS DMEM containing siRNA, DMSO, PP2, or AZD0530. Bottom chambers were filled with 10% FBS DMEM. After 24 h, cells in the top chamber were removed with cotton swabs, and cells on the bottom side of the insert were fixed with 3.7% formaldehyde, stained with 0.5% methylene blue, and counted on an IX50 microscope (Olympus).

Statistical analysis. All experiments were performed at least thrice; values represent the mean of triplicate samples and SEs of the mean.

Figure 3. Identification of an *Src*-responsive region in the *Id1* promoter. **A**, A549 cells were transfected for 24 h with ID1pGL firefly luciferase reporter plus SV40pRL *Renilla* luciferase coreporter followed by incubation for 24 h with DMSO, PP2, or AZD0530 at the concentrations indicated. Triplicate samples were measured using the dual luciferase reporter assay. **B**, A549 cells were triple-transfected with ID1pGL reporter, SV40pRL coreporter plus wild-type (*wt*) *Src*, constitutively active (*ca*) *Src*, or dominant-negative (*dn*) *Src*. Cells were then incubated in 10% FBS DMEM for 18 h followed by serum starvation for 6 h. **C**, A549 cells were triple-transfected for 24 h with constitutively active *Src*, and SV40pRL coreporter plus either the ID1 full-length promoter reporter construct or one of the *Id1* promoter deletion reporter constructs (5' *del-1* to 5' *del-7*). Cells were then incubated in 10% FBS DMEM for 18 h followed by serum starvation for 6 h.



Results

Id gene expression is down-regulated by Src inhibition in cancer cells. To identify novel downstream effectors of Src signaling in cancer, we performed genome-wide expression profiling of cells subjected to Src kinase inhibition. To this end, A549 lung adenocarcinoma cells were incubated for 24 hours with AZD0530 (750 nmol/L) in DMSO or with DMSO alone followed by RNA extraction and expression analysis using Affymetrix U133 plus 2.0 arrays. A total of 175 genes were differentially regulated (≥ 2 -fold) in response to incubation with AZD0530. The genes most dramatically down-regulated by AZD0530 were the inhibitors of differentiation gene family members (ID1-4), inhibitory Smads (SMAD6 and SMAD7), TGF β 1, and SERPINE1/PAI-1 (Supplementary Fig. S1). These findings were confirmed by standard reverse transcription PCR (RT-PCR), and the specificity of the effect of AZD0530 was shown by the fact that levels of Src and of 18S rRNA transcripts were unchanged (Supplementary Fig. S2). Taken together, these expression changes suggested that AZD0530-mediated Src inhibition leads to suppression of the Smad-Id signaling pathway.

Based on its (a) strong association with cancer progression and (b) well-defined regulation by Smad signaling, we chose to focus on better defining the mechanism responsible for Id1 as a target of Src inhibition. To characterize further the effect of AZD0530 on Id1 mRNA levels, A549 cells were incubated with increasing concentrations of AZD0530 (1 nmol/L–10 μ mol/L) for 24 hours.

Quantitative real-time RT-PCR showed a dose-dependent reduction of Id1 expression by AZD0530, which reached a nadir at 100 nmol/L (Fig. 1A). Western blot analysis of companion cultures treated with AZD0530 for 48 hours was performed to show a correlation between AZD0530-mediated Src inhibition and down-regulation of Id1. The results confirmed a dose-dependent reduction in Id1 expression that corresponded with decreased levels of activated pY419-Src, whereas the levels of total Src were unchanged (Fig. 1B). The anti-phospho-Src family antibody detected multiple bands between 55 and 70 kDa, consistent with the presence of multiple Src family members in A549 cells. Reprobing with Src-specific antibody confirmed that the 60-kDa band represented the phosphorylated pp60^{c-Src} protein.

To investigate the functional relationship between Src and Id1 and its therapeutic implications, we examined a panel of human cancer cell lines from four types of cancer, including lung (A549 and H460), breast (T47D and MDA-MB231), prostate (LNCaP and PC-3), and colon (HCT-116 and HT-29), each represented by a pair of cell lines with different invasive potential (36–39). Protein extracts of cells grown under normal conditions were subjected to Western blotting for pY419-Src, total Src, Id1, and actin (Fig. 1C). Immunoblot analysis showed that basal Src activity (pY419-Src) was easily detectable in six of eight cell lines. The levels of pY419-Src corresponded with the reported invasive potential in each pair. Similarly, Id1 levels corresponded with the reported invasive potential and level of pY419-Src level in six of eight cell lines.

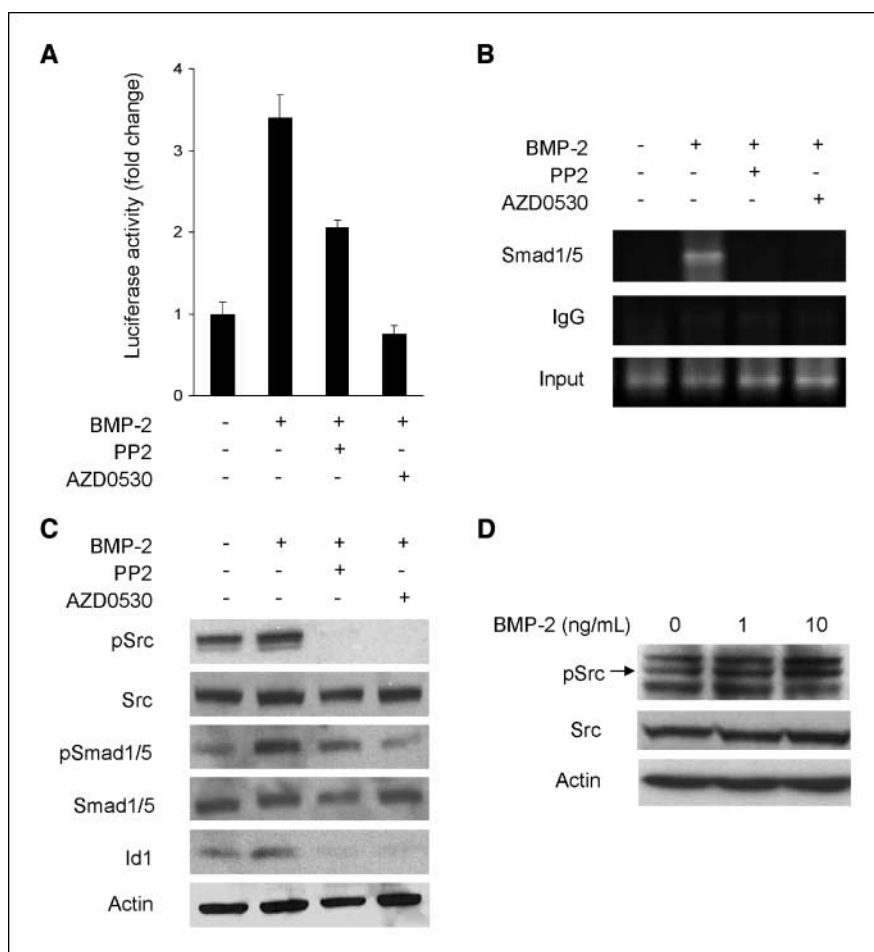
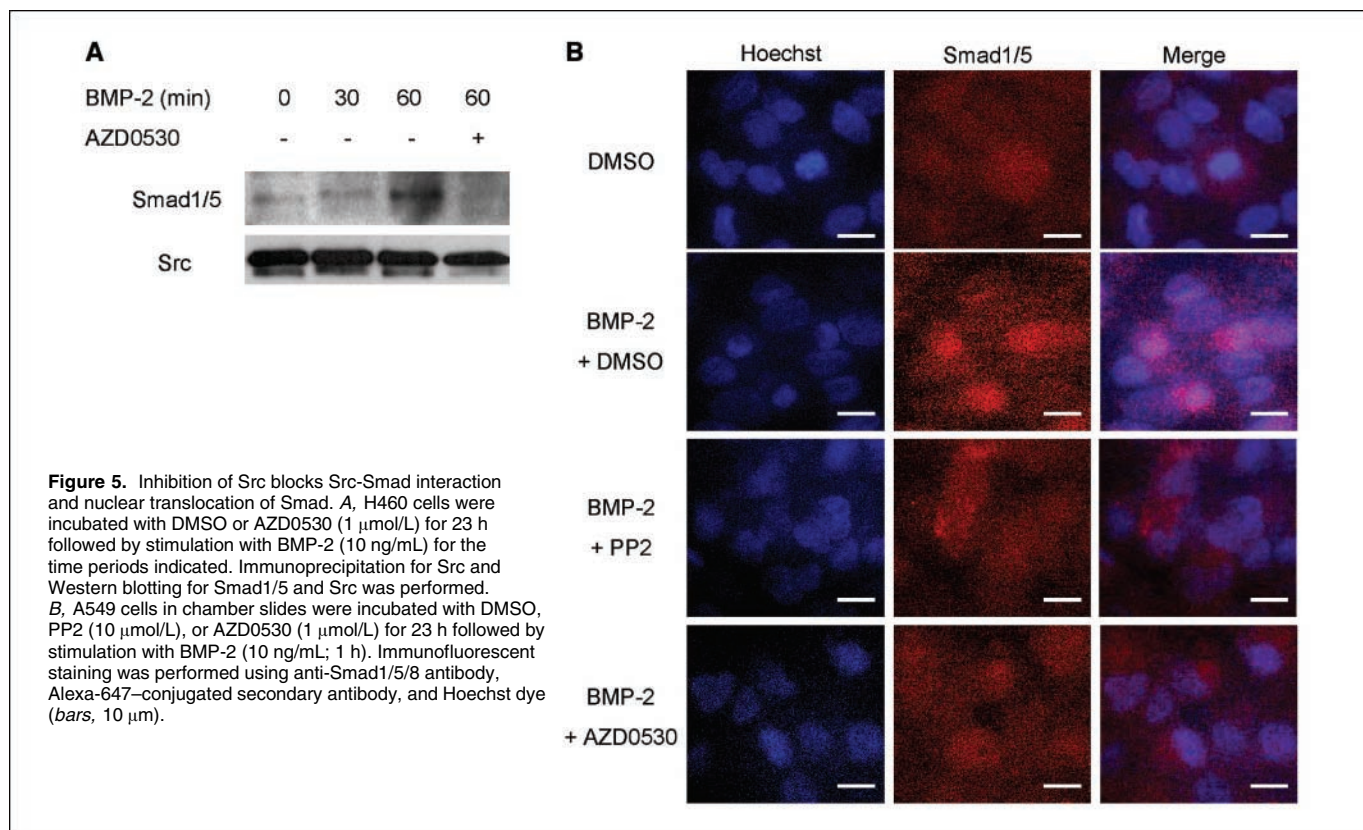


Figure 4. Cooperation of Src with the BMP/Smad signaling pathway. **A**, A549 cells were transfected for 24 h with ID1pGL reporter and SV40pRL coreporter followed by incubation with DMSO, PP2 (10 μ mol/L), or AZD0530 (1 μ mol/L) for 23 h and then stimulated with BMP-2 (10 ng/mL; 1 h). Triplicate samples were analyzed using the dual luciferase reporter system. **B**, A549 cells were incubated with DMSO, PP2 (10 μ mol/L), or AZD0530 (1 μ mol/L) for 23 h followed by stimulation with BMP-2 (10 ng/mL; 1 h). ChIP was performed using anti-Smad1/5 antibody or normal rabbit IgG. DNA was extracted and the proposed Src-responsive region of the Id1 promoter was amplified by PCR. **C**, A549 cells were incubated for 23 h with DMSO, PP2 (10 μ mol/L), or AZD0530 (1 μ mol/L) followed by stimulation with BMP-2 (10 ng/mL; 1 h). Western blotting was performed to determine the levels of pY419-Src, Src, phospho-Smad1/5, Smad1/5, Id1, and actin. **D**, A549 cells were incubated for 1 h with BMP-2 at the concentrations indicated. Western blotting was performed to determine the levels of pY419-Src, Src, and actin.



Although Id1 was highly expressed in the colon cancer cells HCT-116 and HT-29, an inverse relationship between pY419-Src and Id1 levels was observed. This suggests that Src is a major regulator of Id1 but that other pathways exist to activate Id1. Next, the effect of Src inhibition on Id1 expression in each cell line was determined (Fig. 1D). Incubation of the cells with PP2 (10 μ mol/L) or AZD0530 (1 μ mol/L) for 48 hours reduced the levels of pY419-Src and Id1 in all cell lines. These results showed an association between Src and Id1 expression, and that Src inhibitors can effectively mediate Id1 down-regulation.

Reduction of Id1 mRNA and protein levels by Src siRNA and blocking peptides. In addition to small-molecule inhibitors, two alternative approaches to Src-targeting were used to confirm the role of Src in the regulation of Id1 expression. In the first approach, A549 cells were transfected with an Src-specific siRNA pool, as well as an Id1-specific siRNA and a nontargeting control siRNA. After 24 hours, RNA and protein was isolated and RT-PCR and Western blotting was performed, respectively (Fig. 2A and B). The expected effect of each siRNA on its respective target was confirmed. Moreover, Src siRNA also reduced Id1 mRNA and protein levels. The levels of 18S rRNA and actin protein remained unchanged. Src siRNA had no visual effect on other members of the Src family tested (Supplementary Fig. S3). In the second approach, A549 cells were incubated for 24 hours with cell-permeable Src-blocking peptides CpraYKYY- β Ala-k7 and CpraYKYY- β Ala-r7 at the concentrations indicated (Fig. 2C and D; ref. 31). Target specificity of these peptides has been shown earlier (31). Both peptides reduced Id1 transcript levels in a dose-dependent manner. Western blotting showed a reduction in pY419-Src levels and Id1 protein levels, whereas total Src levels remained unchanged. These results

confirmed that inhibition of Src activity or expression leads to reduced Id1 levels.

Identification of an Src-responsive region in the human Id1 promoter. To define further the role of Src in the regulation of Id1 expression, we used dual-luciferase reporter assays to monitor the activity of the Id1 promoter. A549 cells were cotransfected with ID1pGL firefly luciferase reporter (34) and SV40pRL *Renilla* luciferase coreporter for 24 hours followed by incubation with DMSO, PP2, or AZD0530 for an additional 24 hours. As a control, serum starvation for 24 hours decreased the Id1 promoter signal by 54% (Fig. 3A). In comparison, both PP2 and AZD0530 significantly reduced the Id1 promoter signal in a dose-dependent manner. Of note, AZD0530 (1 μ mol/L) in serum-stimulated A549 cells reduced Id1 promoter activity by >90%, suggesting that the promoter is strongly Src dependent. To investigate this further, we tested if Id1 promoter activity could be modulated by enforced expression of wild-type or mutant forms of Src. Under conditions of serum-deprivation, a constitutively active Src mutant resulted in a 3.7-fold induction in Id1 promoter activity, compared with a 3.2-fold induction by serum (Fig. 3B). In contrast, wild-type Src did not significantly alter Id1 promoter activity, whereas dominant-negative Src decreased endogenous (serum deprived) Id1 promoter activity by 85%. In an effort to map the region in the Id1 promoter that was responsible for Src-mediated activation, each of a set of 5'-deletion constructs (5'*del-1* to 5'*del-7*) generated from the full-length (2.2 kbp) Id1 promoter and cloned into the pGL-luciferase reporter (34) were cotransfected with SV40pRL coreporter plus constitutively active Src (Fig. 3C). Again under conditions of serum deprivation, the greatest decrease in the Id1 promoter signal occurred with construct 5'*del-3* (60% of the signal of the full-length

promoter construct), corresponding to a region between positions 1,360 and 1,199 in the Id1 promoter. Analysis of the sequence of this newly characterized Src-responsive region revealed the CAGC motif (positions 1,352–1,349), representing a putative Smad-binding element.

Cross-talk between Src and BMP-Smad signaling pathway. BMP-2 is a well-known inducer of Id1 via Smad binding to consensus elements within the Id1 promoter. The findings above suggest that Src is required for Smad-mediated Id1 activation. To address this, we examined the effect of Src inhibitors on BMP-induced signaling and Id1 expression. In reporter assays, stimulation of A549 cells with BMP-2 (10 ng/mL; 1 h) induced a 3.4-fold increase in Id1 promoter activity above basal, serum-stimulated activity (Fig. 4A). Incubation with PP2 (10 μ mol/L) or AZD0530 (1 μ mol/L) reduced BMP-mediated Id1 promoter activity by 40% or completely blocked the response, respectively. Chromatin immunoprecipitation (ChIP) of unstimulated and BMP-stimulated A549 cells was performed using Smad1/5 antibody followed by DNA extraction and PCR using primers spanning the newly identified Src-responsive region of the Id1 promoter (Fig. 4B). BMP-2 (10 ng/mL; 1 hour) markedly induced Smad binding to the Src-responsive region of the Id1 promoter, and this process was completely blocked in the presence of PP2 (10 μ mol/L) or AZD0530 (1 μ mol/L). Consistently, BMP-induced Smad1/5 phosphorylation and Id1 expression was inhibited by PP2 and AZD0530 (Fig. 4C). This experiment also suggested Src activation by BMP-2, and indeed, a separate experiment confirmed that BMP-2 increased pY419-Src levels in a dose-dependent manner (Fig. 4D). Analysis of the blots with Scion Image software (Scion Corp.) and normalization for total Src revealed an average increase of the pY419-Src levels by 26% to 28% compared with baseline (data not shown).

To determine a physical interaction between Src and Smad, and to confirm the findings in another cell line, H460 lung cancer cells were stimulated with BMP-2 (10 ng/mL) for 30 or 60 minutes followed by immunoprecipitation of Src and Western blotting for Smad1/5 and Src (Fig. 5A). The amount of Smad1/5 present in immune complexes was increased in protein lysates from cells stimulated with BMP-2 (10 ng/mL; 1 hour), and this was blocked in the presence of AZD0530 (1 μ mol/L). Interestingly, a small (3–5 kDa) shift in the size of the Smad protein band occurred by stimulation with BMP-2, suggesting that phosphorylated Smad is recruited into the complex with Src. Next, the effect of Src inhibition on Smad1/5 nuclear translocation was studied. A549 cells were grown on chamber slides and incubated for 23 hours with DMSO, PP2 (10 μ mol/L), or AZD0530 (1 μ mol/L). Cells were then stimulated with BMP-2 (10 ng/mL; 1 hour). Immunofluorescent staining for Smad1/5 was performed and Hoechst dye used to visualize the nuclei (Fig. 5B). In unstimulated cells, Smad1/5 localization was primarily cytoplasmic. Although BMP-2 induced prominent nuclear accumulation of Smad1/5 in ~30% of the cells, which was consistent with a previous report (40), PP2 and AZD0530 almost completely blocked Smad nuclear translocation. In summary, the data showed the existence of cross-talk between Src and Smad pathways, and that Src is involved in BMP-2-mediated Smad activation and nuclear translocation.

Involvement of Id1 in cancer cell invasion. The cellular consequence of the Src-Id1 interaction was investigated by determining the effects of Id1 modulation on the invasiveness of A549 lung carcinoma cells. First, the efficacy of different methods of Id1 and Src antagonism to inhibit invasion was examined (Fig. 6A). Id1 siRNA reduced invasion by 50%, whereas PP2

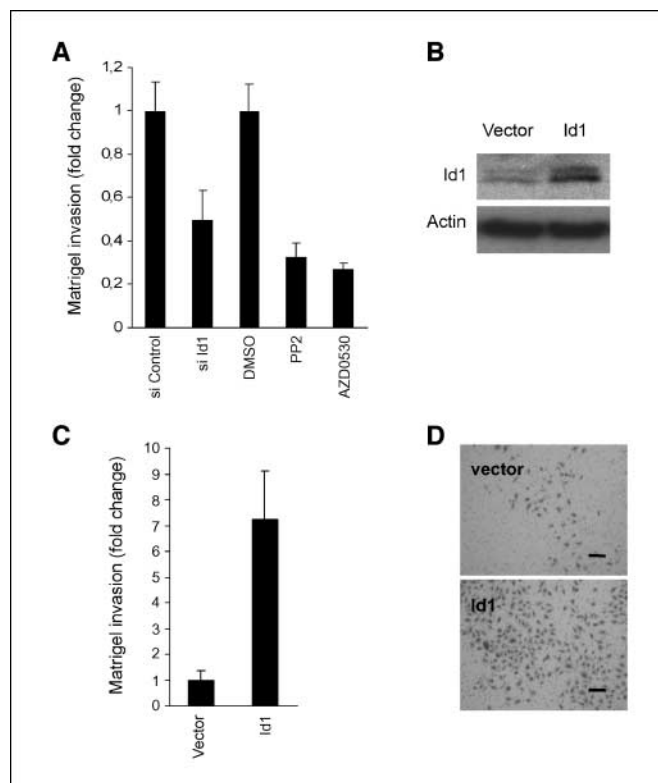


Figure 6. Involvement of Id1 in lung cancer cell invasion. **A**, Matrigel invasion assays were performed in triplicates using A549 cells incubated for 24 h with control si RNA, Id1 siRNA (100 nmol/L), DMSO, PP2 (10 μ mol/L), or AZD0530 (1 μ mol/L). After 24 h, cells in the top chambers were removed, and cells in the bottom chambers were fixed with formaldehyde, stained with methylene blue, and counted. **B**, Id1 overexpressing A549 cells (*Id1*) and empty vector control cells (*vector*) were generated by retroviral infection and Geneticin selection as described under Materials and Methods. Western blotting was performed to determine the levels of Id1 and actin. Next, Matrigel invasion assays were performed in triplicates (**C** and **D**). After 24 h, cells in the upper chambers were removed, and cells in the bottom chambers were fixed with formaldehyde, stained with methylene blue, and counted (*bars*, 50 μ m).

(10 μ mol/L) and AZD0530 (1 μ mol/L) reduced invasion by 68% and 73%, respectively. Next, the dominant role of Id1 in conferring a more aggressive phenotype was tested by generating Id1-overexpressing A549 cells (A549-Id1), using retroviral gene transfer. Western blotting confirmed that Id1 expression was increased markedly in A549-Id1 cells compared with empty vector control cells (Fig. 6B). Matrigel assays revealed that Id1 overexpression enhanced invasion at 24 hours by 7.2-fold compared with vector control (Fig. 6C and D). These results confirmed the involvement of Id1 in lung cancer cell invasion.

Discussion

The connection between BMP-2, Smad1, Id1, and cancer cell invasion is well-established. BMP-2 was found to be overexpressed in primary human lung cancer compared with normal tissue (18) and was shown to activate Smad1/5, to increase Id1 expression, and to promote invasion in lung cancer cells (40, 41). Critical regulatory elements in the Id1 promoter include a BMP-responsive region, a serum-responsive region, and a region associated with constitutive expression in breast cancer cells (14–16, 34). Consistent with previous data from noncancerous cells, the present

study implicates the crosstalk of Src tyrosine kinase signaling with the BMP-Smad pathway as an additional regulator of Id1 expression in cancer (42–45). Importantly, this was also associated with a substantial diminution in invasion. This was shown by the ability of multiple methods of Src inhibition (i.e., small-molecule antagonists, peptide inhibitors, and siRNA) to markedly reduce Id1 expression and promoter activity. Conversely, transient, enforced expression of a constitutively active Src mutant induced the Id1 promoter signal independently of serum or BMP-2. Using a series of Id1 promoter deletion constructs, we identified a novel Src-responsive region in the human Id1 promoter. This region contains the Smad-binding motif CAGC, and we provided evidence for the binding of Smad1/5 to the Src-responsive region. Consistent with the demonstration of a functional interaction between Src and BMP-Smad1/5, and temporal association of BMP-mediated activation of Src and Smad1/5, the formation of a signaling complex of Src and Smad1/5 was shown by coimmunoprecipitation experiments. Further work is expected to reveal the molecular mechanisms by which Src is recruited to the BMP receptor complex and is activated in response to BMP signaling, and by which Src may activate Smad1/5. In this context, previous work by others showed that PP1 (and to a lesser extent, PP2) significantly inhibited TGF β receptor kinase activity and blocked subsequent Smad2/3 signaling; this suggested that some effects seen in our study may have resulted from direct inhibition of TGF β receptor kinase by the small-molecule kinase inhibitors used (46). We used several different molecular approaches to Src targeting (RNA interference, small molecules, dominant-negative mutant, and inhibitory peptides) and the consistent results make it unlikely that off-target effects account for the main observations. Supporting this view, other studies showed that TGF β did not activate, but rather inhibited, Id1 expression (14, 29, 47). Taken together, these data suggest a model of balanced Id1 regulation in which BMP acts positively on Id1 transcription via Smad1/5 and TGF β acts as negative regulator via Smad2/3. Aberrant activation of Src may shift the balance toward increased Smad1/5 signaling, resulting in Id1 overexpression. However, the present report also supports the

existence of other undefined mechanisms of Id1 regulation because HT-29 and HCT-116 cells lacked a positive association between Src activation and Id1 expression. Because missense mutations in Smad4 exist in some colorectal cancer cells, including HT-29, Id1 expression may be driven by Smad-independent pathways in these cells (48). In this regard, the observation that Src inhibition reduced Id1 levels in MDA-MB231 cells, which constitutively express high levels of Id1 in a serum-independent manner, is encouraging (34).

Our study has several clinical implications. First and most important, it points toward a new molecular mechanism of action for Src inhibitors and suggests the use of Id1 as a biomarker. In line with this notion are gene expression signatures in breast and lung cancer, which include both Id1 and Src, and which are associated with tumor aggressiveness and responsiveness to Src inhibition, respectively (49, 50). Second, we speculate that therapeutic strategies based on Src inhibitor-mediated Id1 down-regulation may reduce tumor recurrence, angiogenesis, and metastasis. Beyond this, based on the implication of Src and BMP in osteogenesis, the findings described in the present report may lead to advances in the biology and treatment of malignant and nonmalignant bone disease.

Acknowledgments

Received 11/27/2007; revised 2/5/2008; accepted 2/8/2008.

Grant Support: The Swiss Foundation for Medical-Biological Grants/Swiss National Science Foundation (1184/PASMA-108925; O. Gautschi) and the Swiss Cancer League/Oncosuisse (BIL OCS 01949-08-2006).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank AstraZeneca Pharmaceuticals (Macclesfield, UK) for AZD0530, Dr. Kit Lam (University of California Davis Cancer Center, Sacramento, California) for the Src-blocking peptides, and Dr. Don J. Fujita (Department of Biochemistry and Molecular Biology, University of Calgary, Alberta, Canada) and Dr. June Zhou (UC Davis Cancer Center) for the Src expression constructs; Dr. Joy Yang (Department of Urology, UC Davis) and Ryan R. Davis (Gene Expression Resource, UC Davis Cancer Center) for the technical assistance; and the University of California Davis Cancer Center Gene Expression Resource, supported by Cancer Center Support Grant P30 CA93373-01 from the National Cancer Institute, for the microarray analysis.

References

- Parsons SJ, Parsons JT. Src family kinases, key regulators of signal transduction. *Oncogene* 2004;23:7906–9.
- Summy JM, Gallick GE. Treatment for advanced tumors: SRC reclaims center stage. *Clin Cancer Res* 2006;12:1398–401.
- Hiscox S, Morgan L, Green TP, Barrow D, Gee J, Nicholson RL. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. *Breast Cancer Res Treat* 2006;97:263–74.
- Lee D, Gautschi O. Clinical development of SRC tyrosine kinase inhibitors in lung cancer. *Clin Lung Cancer* 2006;7:381–4.
- Yokota Y. Id and development. *Oncogene* 2001;20:8290–8.
- Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* 1990;61:49–59.
- Sun XH, Copeland NG, Jenkins NA, Baltimore D. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Mol Cell Biol* 1991;11:5603–11.
- Lyden D, Young AZ, Zagzag D, et al. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature* 1999;401:670–7.
- Desprez PY, Hara E, Bissell MJ, Campisi J. Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein Id-1. *Mol Cell Biol* 1995;15:3398–404.
- Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 2003;115:281–92.
- Belletti B, Prisco M, Morrione A, Valentinis B, Navarro M, Baserga R. Regulation of Id2 gene expression by the insulin-like growth factor I receptor requires signaling by phosphatidylinositol 3-kinase. *J Biol Chem* 2001;276:13867–74.
- Lasorella A, Nosedà M, Beyna M, Yokota Y, Iavarone A. Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. *Nature* 2000;407:592–8.
- Ohtani N, Zebede Z, Huot TJ, et al. Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. *Nature* 2001;409:1067–70.
- Korchynski O, ten Dijke P. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J Biol Chem* 2002;277:4883–91.
- Lopez-Rovira T, Chaux E, Massague J, Rosa JL, Ventura F. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. *J Biol Chem* 2002;277:3176–85.
- Katagiri T, Imada M, Yanai T, Suda T, Takahashi N, Kamijo R. Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. *Genes Cells* 2002;7:949–60.
- Kang Y, Chen CR, Massague J. A self-enabling TGF β response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* 2003;11:915–26.
- Langenfeld EM, Calvano SE, Abou-Nukta F, Lowry SF, Amenta P, Langenfeld J. The mature bone morphogenetic protein-2 is aberrantly expressed in non-small cell lung carcinomas and stimulates tumor growth of A549 cells. *Carcinogenesis* 2003;24:1445–54.
- Ling MT, Lau TC, Zhou C, et al. Overexpression of Id-1 in prostate cancer cells promotes angiogenesis through the activation of vascular endothelial growth factor (VEGF). *Carcinogenesis* 2005;26:1668–76.
- Wilson JW, Deed RW, Inoue T, et al. Expression of Id helix-loop-helix proteins in colorectal adenocarcinoma correlates with p53 expression and mitotic index. *Cancer Res* 2001;61:8803–10.
- Schindl M, Oberhuber G, Obermair A, Schoppmann SF, Karner B, Birner P. Overexpression of Id-1 protein is a marker for unfavorable prognosis in early-stage cervical cancer. *Cancer Res* 2001;61:5703–6.
- Schindl M, Schoppmann SF, Strobel T, et al. Level of Id-1 protein expression correlates with poor differentiation, enhanced malignant potential, and

- more aggressive clinical behavior of epithelial ovarian tumors. *Clin Cancer Res* 2003;9:779–85.
23. Tournay O, Benezra R. Transcription of the dominant-negative helix-loop-helix protein Id1 is regulated by a protein complex containing the immediate-early response gene Egr-1. *Mol Cell Biol* 1996;16:2418–30.
 24. Tepper CG, Gregg JP, Shi XB, et al. Profiling of gene expression changes caused by p53 gain-of-function mutant alleles in prostate cancer cells. *Prostate* 2005; 65:375–89.
 25. Swarbrick A, Akerfeldt MC, Lee CS, et al. Regulation of cyclin expression and cell cycle progression in breast epithelial cells by the helix-loop-helix protein Id1. *Oncogene* 2005;24:381–9.
 26. Lin CQ, Singh J, Murata K, et al. A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. *Cancer Res* 2000;60:1332–40.
 27. Desprez PY, Lin CQ, Thomasset N, Simpson CJ, Bissell MJ, Campisi J. A novel pathway for mammary epithelial cell invasion induced by the helix-loop-helix protein Id-1. *Mol Cell Biol* 1998;18:4577–88.
 28. Perk J, Gil-Bazo I, Chin Y, et al. Reassessment of id1 protein expression in human mammary, prostate, and bladder cancers using a monospecific rabbit monoclonal anti-id1 antibody. *Cancer Res* 2006;66: 10870–7.
 29. Tang B, Yoo N, Vu M, et al. Transforming growth factor- β can suppress tumorigenesis through effects on the putative cancer stem or early progenitor cell and committed progeny in a breast cancer xenograft model. *Cancer Res* 2007;67:8643–52.
 30. Fong S, Itahana Y, Sumida T, et al. Id-1 as a molecular target in therapy for breast cancer cell invasion and metastasis. *Proc Natl Acad Sci U S A* 2003;100:13543–8.
 31. Kamath JR, Liu R, Enstrom AM, Lou Q, Lam KS. Development and characterization of potent and specific peptide inhibitors of p60c-src protein tyrosine kinase using pseudosubstrate-based inhibitor design approach. *J Pept Res* 2003;62:260–8.
 32. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A* 2001;98:31–6.
 33. Simon P. Q-Gene: processing quantitative real-time RT-PCR data. *Bioinformatics* 2003;19:1439–40.
 34. Singh J, Murata K, Itahana Y, Desprez PY. Constitutive expression of the Id-1 promoter in human metastatic breast cancer cells is linked with the loss of NF-1/Rb/HDAC-1 transcription repressor complex. *Oncogene* 2002;21:1812–22.
 35. Tanaka A, Fujita DJ. Expression of a molecularly cloned human c-src oncogene by using a replication-competent retroviral vector. *Mol Cell Biol* 1986;6:3900–9.
 36. Navab R, Gonzalez-Santos JM, Johnston MR, et al. Expression of chicken ovalbumin upstream promoter-transcription factor II enhances invasiveness of human lung carcinoma cells. *Cancer Res* 2004;64:5097–105.
 37. Irby RB, Malek RL, Bloom G, et al. Iterative microarray and RNA interference-based interrogation of the SRC-induced invasive phenotype. *Cancer Res* 2005;65: 1814–21.
 38. Chuan YC, Pang ST, Cedazo-Minguez A, Norstedt G, Pousette A, Flores-Morales A. Androgen induction of prostate cancer cell invasion is mediated by ezrin. *J Biol Chem* 2006;281:29938–48.
 39. Gordon LA, Mulligan KT, Maxwell-Jones H, Adams M, Walker RA, Jones JL. Breast cell invasive potential relates to the myoepithelial phenotype. *Int J Cancer* 2003;106:8–16.
 40. Langenfeld EM, Kong Y, Langenfeld J. Bone morphogenetic protein 2 stimulation of tumor growth involves the activation of Smad-1/5. *Oncogene* 2006;25:685–92.
 41. Langenfeld EM, Kong Y, Langenfeld J. Bone morphogenetic protein-2-induced transformation involves the activation of mammalian target of rapamycin. *Mol Cancer Res* 2005;3:679–84.
 42. Wong WK, Knowles JA, Morse JH. Bone morphogenetic protein receptor type II C-terminus interacts with c-Src: implication for a role in pulmonary arterial hypertension. *Am J Respir Cell Mol Biol* 2005;33:438–46.
 43. Mima A, Matsubara T, Arai H, et al. Angiotensin II-dependent Src and Smad1 signaling pathway is crucial for the development of diabetic nephropathy. *Lab Invest* 2006;86:927–39.
 44. Kersten C, Dosen G, Myklebust JH, et al. BMP-6 inhibits human bone marrow B lymphopoiesis-upregulation of Id1 and Id3. *Exp Hematol* 2006;34:72–81.
 45. Hagen M, Fagan K, Steudel W, et al. Interaction of interleukin-6 and the BMP pathway in pulmonary smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1473–9.
 46. Maeda M, Shintani Y, Wheelock MJ, Johnson KR. Src activation is not necessary for transforming growth factor (TGF)- β -mediated epithelial to mesenchymal transitions (EMT) in mammary epithelial cells. PP1 directly inhibits TGF- β receptors I and II. *J Biol Chem* 2006;281:59–68.
 47. Murillo MM, del Castillo G, Sanchez A, Fernandez M, Fabregat I. Involvement of EGF receptor and c-Src in the survival signals induced by TGF- β 1 in hepatocytes. *Oncogene* 2005;24:4580–7.
 48. Woodford-Richens KL, Rowan AJ, Gorman P, et al. SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway. *Proc Natl Acad Sci U S A* 2001;98:9719–23.
 49. Minn AJ, Gupta GP, Padua D, et al. Lung metastasis genes couple breast tumor size and metastatic spread. *Proc Natl Acad Sci U S A* 2007;104:6740–5.
 50. Bild AH, Yao G, Chang JT, et al. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature* 2006;439:353–7.