

# Src Family Kinase Activity Is Required for Murine Embryonic Stem Cell Growth and Differentiation

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## ABSTRACT

Self-renewal and differentiation of embryonic stem (ES) cells are regulated by cytokines and growth factors through tyrosine kinase-dependent signaling pathways. In murine ES cells, signals for self-renewal are generated by the leukemia inhibitory factor (LIF). LIF and other growth factors are linked to the activation of the Src family of cytoplasmic protein-tyrosine kinases (SFKs), which consists of eight members having shared structural architecture. In this article, we show that murine ES cells express seven SFKs, three of which (Hck, Src, and Fyn) exhibit constitutive activity in self-renewing ES cells. Differentiation of ES cells to embryoid bodies was associated with rapid transcriptional silencing of Hck and Lck and with the loss of the corresponding kinase proteins. The expression of other family members remained relatively constant, although some loss of Fgr and Lyn proteins was

observed during differentiation. Like ES cells, embryoid bodies maintained constitutive Src and Fyn kinase activity. Partial inhibition of endogenous SFK activity with the ATP-competitive inhibitors 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine or Src kinase inhibitor-1 induced differentiation of ES cells in the presence of LIF. In contrast, suppression of all SFK activity with higher concentrations of these inhibitors, or with the more potent compound A-419259 (*Bioorg Med Chem Lett* 12:1683–1686, 2002) blocked differentiation in response to LIF withdrawal. It is surprising that these inhibitor-treated cells remained pluripotent despite the absence of LIF. Our results implicate individual members of the Src kinase family in distinct ES cell renewal and differentiation pathways and show that small-molecule SFK inhibitors can control ES cell fate.

Maintenance of embryonic stem (ES) cell pluripotency in culture requires the suppression of differentiation by self-renewal signaling pathways. In murine ES cells, addition of the cytokine leukemia inhibitory factor (LIF) to the culture medium is required to maintain pluripotency (Smith et al., 1988; Williams et al., 1988). LIF, signaling through a gp130/LIF receptor complex activates multiple cytoplasmic protein-tyrosine kinases, including members of the Jak and Src families (Burdon et al., 1999; Hirano et al., 2000; Smithgall et al., 2000). Kinase activation induces phosphorylation of the Stat3 transcription factor downstream, which is both necessary and sufficient for self-renewal (Niwa et al., 1998; Mat-

suda et al., 1999). Suppression of Jak signaling with antisense RNA or using selective pharmacological inhibitors blocks Stat3 activation and induces ES cell differentiation, suggesting that Jak kinase activity is essential for LIF-induced self-renewal through the Stat3 pathway (Ernst et al., 1996; Chambers et al., 2003).

Of the eight mammalian Src family kinases (SFKs), only Hck and Yes have been studied in detail in the context of murine ES cells. ES cells carrying a targeted activating mutation in one *hck* allele showed a reduced LIF requirement for self-renewal, suggesting that Hck plays a role in the maintenance of pluripotency (Ernst et al., 1994). More recently, Ernst et al. (1999) established a correlation between the ability of truncated gp130 mutants to activate Hck and suppress ES cell differentiation. Mutant gp130 also failed to activate Stat3, suggesting a connection between Hck and Stat3 activation in ES cells. This possibility is supported by

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**ABBREVIATIONS:** ES, embryonic stem; SKI-1, Src kinase inhibitor-1; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; RT, reverse transcriptase; PCR, polymerase chain reaction; SFK, Src family kinase; LIF, leukemia inhibitory factor; IP, immunoprecipitation; IB, immunoblotting; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; SIE, *sis*-inducible element; ALP, alkaline phosphatase; EB, embryoid body; STI-571, imatinib; AG490,  $\alpha$ -cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; SU6656, 2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide).

the direct activation of Stat3 by active mutants of Hck and Src in transformed rodent fibroblasts (Yu et al., 1995; Schreiner et al., 2002). Treatment of murine ES cells with the Src family kinase inhibitor SU6656 (Blake et al., 2000) resulted in ES cell differentiation, also supporting a role for Src family kinases in self-renewal (Anneren et al., 2004). In this same study, suppression of c-Yes using an antisense approach also led to differentiation, implicating Yes as the target for SU6656. On the other hand, work in *Xenopus laevis* embryos has demonstrated a role for the Src kinase family in early germ-layer formation, suggesting a broader role for SFKs in differentiation (Weinstein et al., 1998; Weinstein and Hemmati-Brivanlou, 2001). *X. laevis* Fyn and the unique Src homolog Laloo seem to be key intermediates linking fibroblast growth factor signaling at the cell surface to nuclear events required for mesoderm induction. Together, these findings suggest that individual members of the Src family may control distinct and possibly opposing aspects of early development.

Despite the importance of the Src kinase family to amphibian body patterning, little work has been done to investigate the contribution of these closely related but nonetheless unique kinases to early events in mammalian ES cell differentiation. In this report, we demonstrate that seven of the eight mammalian Src family kinases are expressed in self-renewing murine ES cells. Individual SFKs showed distinct transcriptional changes as ES cells differentiated to embryoid bodies. Fgr, Hck, and Lck expression diminished as a function of differentiation, whereas transcript levels of Src, Fyn, Yes, and Lyn remained constant. Pharmacological inhibition of overall SFK activity with the highly potent antagonist A-419259 [compound 13 from Calderwood et al. (2002)] suppressed ES cell differentiation to embryoid bodies while preserving pluripotency, despite the absence of LIF. Furthermore, this inhibitor did not affect Stat3 activation, suggesting that Src family kinases are not involved in the LIF-Stat3 self-renewal pathway. Complete inhibition of SFK activity with the less potent inhibitors PP2 and Src kinase inhibitor-1 (SKI-1) also blocked differentiation while maintaining pluripotency in a manner similar to that of A-419259. It is interesting that partial inhibition of SFK activity with lower concentrations of these two inhibitors induced differentiation in the presence of LIF. Together, these results suggest that individual members of the Src kinase family control distinct aspects of ES cell pluripotency and differentiation and demonstrate that small-molecule SFK inhibitors can influence ES cell fate.

## Materials and Methods

**Embryonic Stem Cell Culture.** The D3 line of embryonic stem cells was obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, and 1000 U/ml LIF (Chemicon International, Temecula, CA) as described elsewhere (Niwa et al., 1998).

**Immunoprecipitation and Immunoblot Analysis.** Antibodies, applications, and sources include the following: Src phosphospecific for immunoblotting (IB) (pY418), Biosource International, Camarillo, CA; c-Src for immunoprecipitation (IP) and IB, Fyn for IB, Hck for IP and IB, Lck for IP and IB, Lyn for IP and IB, Yes for IP and IB, Fgr for IB, Stat5 for IP and IB, and antiphosphotyrosine PY99 for IB,

all from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); Fyn for IP and Oct3/4 for IB, both from BD Biosciences (San Jose, CA); Fgr for IP, Serotec (Oxford, UK); and actin for IB, Chemicon.

For immunoblot analysis, ES cells ( $10^7$ ) were released from the culture plates with trypsin-EDTA (30 s treatment), washed with ice-cold PBS, and immediately suspended in SDS-polyacrylamide gel electrophoresis loading buffer (0.5 ml). For immunoprecipitation experiments, cells ( $5 \times 10^6$ ) were washed and resuspended in 1 ml of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA) supplemented with 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, 0.25  $\mu\text{l}$  of Benzonase (Novagen, Madison, WI), and a protease inhibitor cocktail (Calbiochem, San Diego, CA). For protein-tyrosine phosphatase treatment, immunoprecipitates were washed with phosphatase buffer (25 mM MOPS, pH 7.0, 50 mM NaCl, 1 mM dithiothreitol, and 0.05% Tween 20) and treated with 25 U of *Yersinia enterocolitica* protein tyrosine phosphatase (Calbiochem) before immunoblot analysis. Additional details of the immunoprecipitation and immunoblotting procedures are described elsewhere (Schreiner et al., 2002).

**Expression of SFK cDNA Clones in 293T Cells.** Full-length cDNA clones for murine Fyn, Hck, Lck, and Yes were isolated from D3 ES cell RNA by RT-PCR and subcloned into the mammalian expression vector pCDNA3.1 (Invitrogen, Carlsbad, CA). Clones for the expression of murine Fgr, Lyn, and Src were obtained from the American Type Culture Collection. Culture and transient transfection of human 293T cells was performed as described elsewhere (Rogers et al., 1996).

**SFK Inhibitors.** SU6656, SKI-1, and PP2 were purchased from Calbiochem. A-419259 was provided by Abbott Bioresearch (Worcester, MA).

**Electrophoretic Mobility Shift Assay for Stat3.** The electrophoretic mobility shift assay (EMSA) for Stat3 activation has been described elsewhere (Schreiner et al., 2002; Choi and Smithgall, 2004). In brief, ES cells were centrifuged and washed once with PBS, and nuclear extracts were made using the Nuclear Extract Kit (Active Motif Inc., Carlsbad, CA). The probe used for the Stat3 EMSA is based on the *sis*-inducible element (SIE). Complementary SIE oligonucleotides (20 pmol) were annealed in 10  $\mu\text{l}$  of 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA and labeled with [ $\alpha$ - $^{32}\text{P}$ ]dATP and the Klenow fragment of DNA polymerase. EMSA was performed with 10  $\mu\text{g}$  of ES cell nuclear extract and 40,000 cpm of labeled SIE probe in a final volume of 20  $\mu\text{l}$ . Reactions were incubated at 30°C for 30 min, separated on 5% polyacrylamide gels, and Stat3-SIE complexes were visualized by autoradiography.

**RT-PCR.** Total RNA was isolated using the Totally RNA Kit (Ambion, Austin, TX). Poly(dT)-primed cDNA was synthesized from 2  $\mu\text{g}$  of total RNA using Moloney murine leukemia virus reverse transcriptase. For semiquantitative analysis, one-fifteenth of each RT reaction was then used in 50  $\mu\text{l}$  of PCR reactions using the primer pairs described in Table 1. Primer sequences for Gata4, AFP, and HPRT are from Fujikura et al. (2002). Aliquots (5  $\mu\text{l}$ ) of each reaction were run on 2% agarose gels and were stained with ethidium bromide.

Quantitative PCR analysis of Hck, Lck, and Oct3/4 expression levels was performed with the Taqman Gene Expression Assays from Applied Biosystems (Foster City, CA) using Mastermix and the manufacturer's protocols. cDNA was prepared as described above, and a final dilution of 1:20 was used. Measurements were made on an ABI 7700 instrument. Expression levels were calculated using RT reactions from self-renewing embryonic stem cells as the calibrator sample and glyceraldehyde-3-phosphate dehydrogenase as the internal control. Changes in the expression of each gene were calculated from cycle counts ( $C_T$ ), where gene expression =  $2^{-\Delta C_T}$ . Insignificant signal was detected in the no-RT and no-template controls.

**Enzyme Assays.** Recombinant full-length human Src, Fyn, and Yes were obtained from Upstate Biotechnology (Lake Placid, NY). Murine Hck was expressed in Sf9 insect cells as an N-terminal hexahistidine fusion protein and was purified as described previously (Schindler et al., 1999). Kinase assays were performed using

the fluorescence resonance energy transfer-based Z'-Lyte Src kinase assay kit according to the manufacturer's instructions (Invitrogen). All assays were performed in triplicate in low-volume, nonbinding 384-well plates (Corning Glassworks, Corning, NY) and read on a Gemini XS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). The assay was first optimized for linearity with respect to incubation time and kinase concentration. The  $K_m$  value for ATP was then determined for each kinase over an ATP concentration range of 0 to 200  $\mu$ M with a saturating concentration of substrate peptide (2  $\mu$ M). The  $K_m$  value was determined by nonlinear regression curve-fitting using the Michaelis-Menten equation and Prism software (GraphPad Software Inc., San Diego, CA).  $IC_{50}$  values were determined for A-419259 and SKI-1 in the presence of an ATP concentration equal to twice the  $K_m$  value for each kinase.  $IC_{50}$  values were calculated using a four-parameter sigmoidal curve fit and Prism software.

**Alkaline Phosphatase Assay.** Alkaline phosphatase (ALP) assays were conducted as described previously (Zandstra et al., 2000). Cells were counted before lysis, and results were expressed as units of ALP activity per 1000 cells.

**Embryoid Body Assays.** Embryoid bodies (EBs) were cultured as described previously (Qu et al., 1997). In brief,  $3.5 \times 10^4$  ES cells were plated in 7 ml of growth medium without LIF in 10-cm bacterial-grade Petri dishes. For quantification, EBs were cultured in the presence of 0.3% methylcellulose and were counted 10 days later.

## Results

**ES Cells Express Multiple Src Family Kinases.** Eight Src family kinase (SFK) gene products have been identified in the mouse: Src, Fyn, Yes, Blk, Fgr, Hck, Lyn, and Lck (Thomas and Brugge, 1997). Although the individual Src family kinases Hck and Yes have been studied in the context of ES cell growth and differentiation (Ernst et al., 1994, 1996, 1999; Anneren et al., 2004), the expression patterns and functions of other SFKs are less clear. To begin to address the question of the role of the SFKs in the regulation of ES cell physiology, we used RT-PCR to identify the SFKs expressed in self-renewing ES cells. ES cells were grown with LIF in a feeder-free system, and total RNA was isolated and subjected to reverse transcription. PCR primers specific for each kinase were designed to span at least one intron (Table 1) and were used to amplify cDNA fragments for each SFK.

RT-PCR analysis revealed that self-renewing mouse ES cells express seven of the eight Src family members, with the sole exception being Blk (Fig. 1A). Amplification of an appropriately sized fragment from genomic DNA using the Blk primers confirmed that the absence of an RT-PCR fragment for Blk was not

caused by ineffective primer design (data not shown). Failure of the HPRT primers to amplify a fragment from control reactions lacking reverse transcriptase confirmed the absence of contaminating genomic DNA in the RNA preparations (Fig. 1A). Finally, to be certain that the observed PCR products were derived from transcripts of the corresponding SFKs, amplified fragments were subjected to nucleotide sequencing. In each case, the sequence corresponded to the expected partial cDNA sequence of each kinase (data not shown).

We next determined whether the transcription of individual SFKs reflected the presence of the corresponding SFK proteins in self-renewing ES cells. As shown in Fig. 1B, each of the kinases identified by RT-PCR was present in ES cell lysates as determined by immunoprecipitation and immunoblotting with kinase-specific antibodies. Antibodies used for immunoblot analysis were raised against epitopes distinct from those used for the immunoprecipitation step and, in a different species, to prevent interference from the antibody heavy chain and to provide a second level of specificity for each kinase (see *Materials and Methods*).

To determine whether SFKs are constitutively active in self-renewing ES cells, each of the kinase immunoprecipitates was also tested for reactivity with the phosphospecific antibody pY418. This antibody recognizes the conserved phosphotyrosine residue in the activation loop of active c-Src and other SFKs (Schreiner et al., 2002; Wilson et al., 2002). Using this antibody, autophosphorylated Hck, Src, and Fyn were observed in self-renewing ES cells (Fig. 1B), suggesting that these kinases are constitutively active. In each case, pY418 antibody immunoreactivity was completely eliminated by protein-tyrosine phosphatase pretreatment of the immunoprecipitated kinase. The remaining SFKs did not show evidence of constitutive activation by this approach.

To ensure that the pY418 antibody is able to recognize the active form of each SFK present in ES cells, recombinant murine Fgr, Fyn, Hck, Lck, Lyn, Src, and Yes were expressed in 293T cells and were subjected to the same immunoprecipitation/immunoblotting procedure. Previous work from our group has established that high-level expression of SFKs induces their autophosphorylation in this cell line (Briggs et al., 2001). As shown in Fig. 1C, pY418 immunoreactivity was observed for each kinase (Fig. 1C). Treatment of the transfected 293T cells with the Src inhibitor A-419259 (see below) before lysis or incubation of the precipitated kinases with a protein-tyrosine phosphatase before immunoblotting resulted in a substantial

TABLE 1

PCR primers used in this study

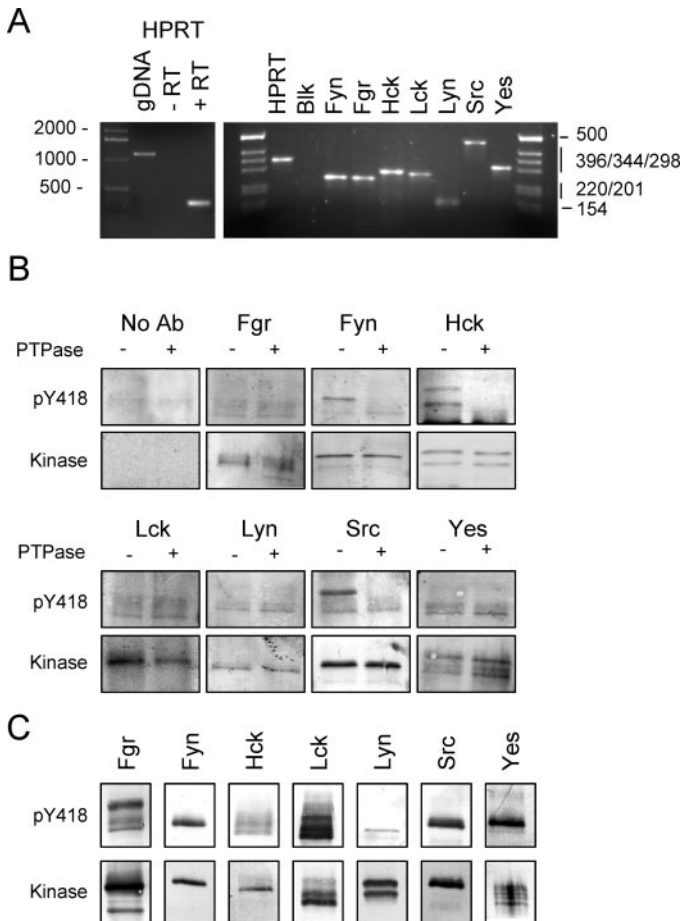
All primer pairs with the exception of Lyn span at least one intron to prevent interference from contaminating genomic DNA. The PCR product sizes (measured in base pairs) expected from each primer pair with the genomic DNA (gDNA) and cDNA templates are shown in the columns on the far right.

Gene	PCR Primer Sequences (5'-3')		PCR Product Size	
	Forward	Reverse	gDNA	cDNA
Hck	gactttgacccccagcagcgagac	cacacttctccaaactgccca	679	274
Fgr	ggtacttcggaagatcagta	cgcactaggtcctgtatggag	407	240
Lyn	gaacaccctggatgctgttt	tgaacctgagtcactcggc	123	123
Yes	ggattatgccacaaagttaa	gacaatataaatgggctctt	472	306
Fyn	ggtactttggaaaacttggc	gtaccagttgctgaagtgtt	1515	239
Lck	cctgacgactggccaagaa	gccaccgttgtctaggtta	756	264
Blk	ctttccctgtcctgtgaaag	catccagacttcgcaaaact	623	294
Src	gccttcaggcatggcctatgt	gtggctcagtgagcgtaaa	808	466
AFP	tcgtattccaaacaggagg	aggcttttgcttcaccag	1226	173
Gata4	gcctgtatgtaatgcctgcg	ccgagcaggaattgaagagg	3363	499
HPRT	ctcgaagtgttgatacag	tggcctataggctcatagt	1187	349



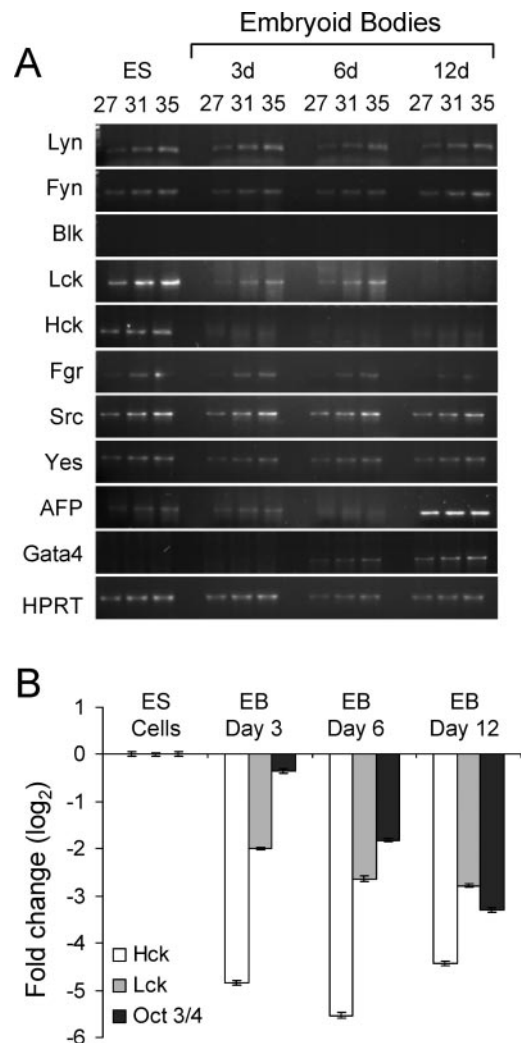
reduction in the pY418 signal, confirming specificity for the active form of each kinase (data not shown).

**Src Family Kinase Expression Profiles in Developing Embryoid Bodies.** EBs are highly organized structures that develop from ES cells grown in suspension culture in the absence of LIF (Keller, 1995). Differentiation of ES cells into EBs involves the loss of pluripotency, the appearance of cells comprising the three germ layers associated with early development, and the eventual appearance of terminally differentiated cell types. Loss of pluripotency and differentiation to EBs is quite rapid, with few pluripotent cells remaining after 6 days of EB development (Qu et al., 1997).



**Fig. 1.** Mouse ES cells express multiple Src family kinases. **A**, RT-PCR analysis of SFK transcripts from murine ES cell total RNA. Total RNA was isolated from self-renewing ES cells grown in the presence of LIF and subjected to RT-PCR (30 cycles) using SFK-specific primers or HPRT as a positive control. See Table 1 for primer sequences and expected sizes of the RT-PCR products. PCR products were separated on agarose gels and were stained with ethidium bromide (right). To control for contaminating genomic DNA (gDNA), RT-PCR reactions were repeated with the HPRT primer set in the presence or absence of RT. A third reaction was included with gDNA as a template to confirm amplification of the larger gDNA PCR product. An image of the agarose gel from this experiment is shown on the left. The lengths of the DNA size markers are indicated in base pairs. **B**, SFKs were immunoprecipitated with isoform-specific antibodies from ES cell lysates. Aliquots were treated with (+) or without (-) *Y. enterocolitica* protein-tyrosine phosphatase before immunoblotting with the activation-specific antibody pY418 or with antibodies specific to each SFK protein (Kinase). A control immunoprecipitation reaction was run without a kinase antibody (No Ab). Blk protein was not detected, consistent with the RT-PCR result (data not shown). **C**, as a positive control, murine SFK cDNA clones were expressed in 293T cells. Individual SFK members were then immunoprecipitated and immunoblotted for pY418 and each kinase protein as in **B**.

As outlined in the Introduction, previous reports have suggested a role for SFKs in the regulation of *X. laevis* early development (Weinstein et al., 1998; Weinstein and Hemmati-Brivanlou, 2001). These observations led us to address the question of how the SFKs might be regulated during differentiation of ES cells into EBs. To address this issue, we first investigated the transcriptional regulation of each SFK during differentiation to EBs using RT-PCR. Single ES cells were seeded in bacterial-grade Petri dishes in the absence of LIF to permit EB formation. EBs were harvested 3, 6, and 12 days later, and RNA was isolated and analyzed for SFK transcripts. Transcription of Gata4 and AFP, two markers of extraembryonic endoderm formation, were assessed to ensure that the EBs were undergoing differentiation (Fujikura et al., 2002). HPRT transcripts were assayed as a positive control at each time point. As shown in Fig. 2A, this analysis revealed three distinct patterns of SFK expression in devel-



**Fig. 2.** Src family kinase expression in developing embryoid bodies. **A**, RT-PCR analysis of individual Src family kinases, Gata4, AFP, and HPRT transcript levels in ES cells and 3-, 6-, and 12-day embryoid bodies. PCR reactions were analyzed after 27, 31, and 35 cycles to permit semi-quantitative analysis. Images of ethidium bromide-stained agarose gels are shown. **B**, quantitative real-time RT-PCR analysis of Hck, Lck, and Oct3/4 expression in developing EBs. Murine glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference, and self-renewing ES cells were used as the calibrator. Data are expressed as log<sub>2</sub> fold changes in expression levels (see Materials and Methods).

oping EBs. Expression of Lyn, Fyn, Src, and Yes remained constant throughout the 12 days of EB development, with transcript levels similar to those seen in self-renewing ES cells. In contrast, Blk expression was not detected at any stage of EB formation. Finally, Fgr, Hck, and Lck transcript levels decreased during EB formation. The decrease in Hck expression was especially rapid, with little transcript detectable by 3 days of EB formation. This observation supports a role for Hck in the regulation of self-renewal or very early events in differentiation. Lck expression decreased more gradually than Hck, with initial decreases at days 3 and 6, followed by complete transcript loss at day 12. In contrast to Hck and Lck, a partial decrease in Fgr transcript levels was observed during EB formation, with transcripts still detectable in 12-day EBs.

We next investigated the decrease in Hck and Lck expression with greater resolution through the use of quantitative real-time RT-PCR. Hck demonstrated a 30-fold decline in transcript levels during the initial 3 days of EB formation, whereas Lck displayed a slower rate of decrease (Fig. 2B). Transcript levels of both Hck and Lck decreased at a faster rate than Oct3/4, a transcription factor expressed in self-renewing ES cells that decreases as a function of differentiation (Niwa et al., 2000). This result provides further evidence that these two SFKs may be involved in signaling pathways that regulate either renewal or the early stages of differentiation to embryoid bodies.

The dynamic nature of transcriptional regulation of individual Src family members provided strong evidence that individual SFKs might have distinct functions in the regulation of ES cell renewal and differentiation. We pursued this question further by testing whether the RT-PCR results were reflected in changes in kinase protein levels and if the activity of individual SFKs varies during early EB development. We first examined the protein levels for Hck and Lck in ES cells and in 6-day EBs using the immunoprecipitation/immunoblotting paradigm described for Fig. 1. As shown in Fig. 3A, levels of Hck and Lck proteins decreased significantly during early EB development, which is in agreement with the RT-PCR data. Note that Oct3/4 expression decreased by day 6, consistent with the loss of pluripotent ES cells from the culture.

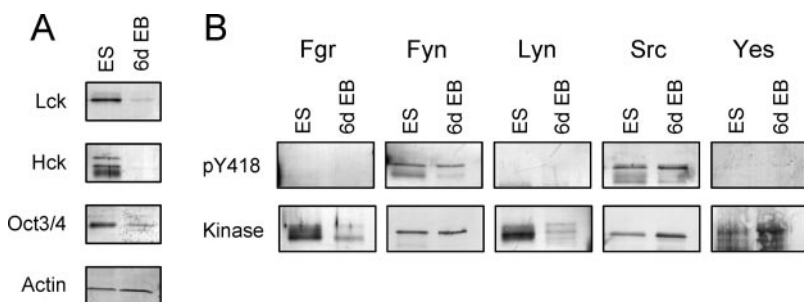
Changes in protein level and autophosphorylation were then evaluated for the remaining SFKs during early EB formation. Fgr, Fyn, Lyn, Src, and Yes were immunoprecipitated from ES cells and 6-day EBs and analyzed for relative levels of kinase protein and activity, as described for Fig. 1. As shown in Fig. 3B, protein levels of Fyn, Src, and Yes did not change markedly as a function of EB formation, whereas Fgr decreased slightly, consistent with the RT-PCR result. It

is interesting that Lyn levels also decreased and demonstrated a change in the ratio of the two isoforms. In terms of activity, both Src and Fyn maintained equivalent immunoreactivity with the pY418 antibody in ES cells and 6-day EBs. In contrast, the other SFKs failed to react with the pY418 antibody in either ES cells or EBs, suggesting that these kinases are not constitutively active under these culture conditions.

**Src Family Kinase Inhibition Blocks ES Cell Differentiation.** The complex changes in SFK gene expression observed during EB formation suggested that the activity of some SFKs might be essential to early development. To test this idea, we used small-molecule inhibitors to determine whether suppression of SFK activity would affect ES cell growth, self-renewal, or differentiation. We first compared four distinct SFK inhibitors (SKI-1, PP2, A-419259, and SU6656) for their effects on ES cell growth under conditions of undifferentiated colony formation in the presence of LIF. As described in more detail below, partial suppression of SFK activity with lower concentrations of inhibitors led to ES cell differentiation in the presence of LIF. However, complete suppression of overall SFK activity suppressed ES cell growth and delayed differentiation while maintaining pluripotency.

The first inhibitor examined was SKI-1, a 4-anilinoquinazoline with moderate potency toward the Src kinase family (Tian et al., 2001). To determine the effect of SKI-1 on overall SFK activity in vivo, self-renewing ES cells were treated with this compound at 5, 10, or 20  $\mu$ M followed by immunoblotting of whole-cell lysates with the pY418 phosphospecific antibody. As shown in Fig. 4A, SKI-1 treatment reduced pY418 immunoreactivity in a dose-dependent fashion, with partial inhibition at 5  $\mu$ M and nearly complete inhibition at 20  $\mu$ M. To determine the biological impact of inhibitor treatment, ES cells were plated in assays for undifferentiated colony formation over the same SKI-1 concentration range in the presence or absence of LIF. As shown in Fig. 4B, control cells grown without inhibitor in the presence of LIF formed large, tight colonies of cells characteristic of undifferentiated ES cells in culture. In the absence of LIF, these colonies rapidly dispersed as the cells underwent differentiation. After 3 days, ES cell colonies treated with the two lower concentrations of SKI-1 (5 and 10  $\mu$ M) dispersed and seemed to differentiate despite the presence of LIF. At 20  $\mu$ M SKI-1, however, ES cell growth and differentiation were blocked, resulting in small colonies independent of the presence of LIF.

The next SFK inhibitor tested was the pyrazolo-pyrimidine PP2, another broad-spectrum inhibitor of SFK activity (Hanke et al., 1996). As shown in Fig. 4A, strong SFK inhibition was observed in ES cells after treatment with 5  $\mu$ M



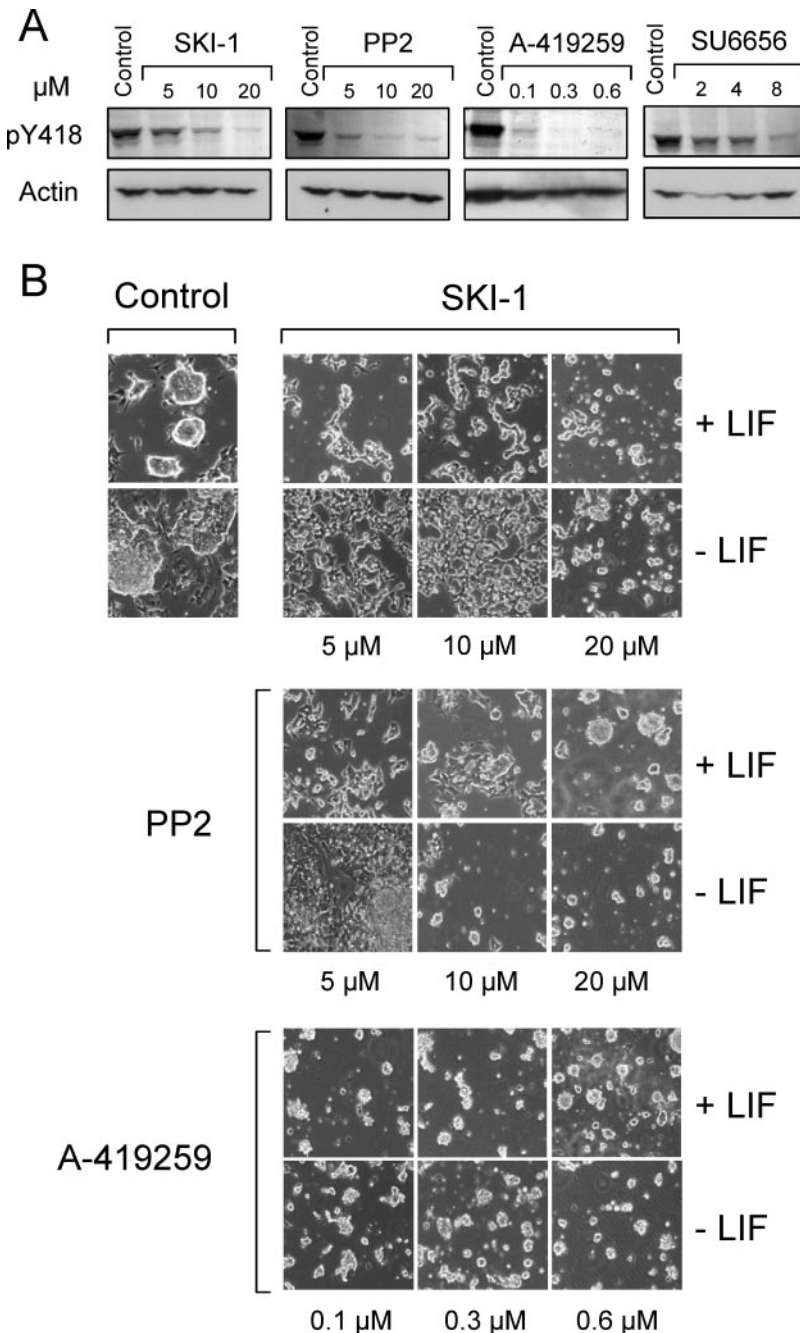
**Fig. 3.** SFK protein levels and activity in developing EBs. A, Hck and Lck were immunoprecipitated from aliquots of ES cell and 6-day EB lysates and were analyzed for protein levels by immunoblotting, as described for Fig. 1B. Oct3/4 and actin protein levels were analyzed by immunoblotting of the cell lysates. B, kinase protein levels and activity (pY418) of Fgr, Fyn, Lyn, Src, and Yes were compared for ES cells and 6-day EBs as described for Fig. 1B.

PP2, with nearly complete inhibition at 10  $\mu\text{M}$ . As observed with SKI-1, partial inhibition of SFK activity with PP2 also promoted the loss of undifferentiated colony morphology in the presence of LIF, whereas complete inhibition correlated with the persistence of small undifferentiated colonies in the presence or absence of LIF. A potential complication of studies using PP2, however, is nonspecific inhibition of other protein-tyrosine kinases, including the PDGF receptor, c-Abl, and c-Kit (Blake et al., 2000; Tatton et al., 2003). To ensure that the phenotype observed with PP2 was not a result of inhibition of these kinases, we treated ES cells with either the platelet-derived growth factor receptor inhibitor AG1295 or the c-Abl and c-Kit inhibitor STI-571 in the presence or absence of LIF. Neither of these inhibitors affected undifferentiated ES cell colony formation, suggesting that the ob-

served effects of PP2 treatment were caused by SFK inhibition (data not shown).

A-419259 displays very high potency toward SFKs both in vitro and in vivo and, unlike PP2, is 1000-fold less potent toward c-Abl in vitro (Wilson et al., 2002). Treatment of ES cells with A-419259 resulted in nearly complete SFK inhibition at 100 nM (Fig. 4A), with complete loss of SFK activity at 300 nM. Treatment of ES cells with these concentrations of A-419259 resulted in the formation of small colonies with an undifferentiated morphology, which is strikingly similar to the effects of PP2 and SKI-1 at concentrations sufficient to suppress all SFK activity (Fig. 4B).

The final inhibitor tested in these studies was SU6656, which may be more specific for individual SFK isoforms than the other inhibitors tested. For example, SU6656 blocks Yes



**Fig. 4.** Inhibition of SFK activity blocks ES cell differentiation. **A**, ES cells were treated with the SFK inhibitors SKI-1, PP2, A-419259, or SU6656 at the concentrations indicated for 24 h. Cell lysates were probed for active SFKs (pY418, top) and actin (bottom) as a loading control by immunoblotting. **B**, ES cells ( $5 \times 10^5$ ) were plated on 60-mm plates and were grown in the presence of LIF for 24 h. The cells were then washed, and SFK inhibitors were added to the indicated concentrations either in the presence or absence of LIF. Photographs were taken 3 days after the addition of inhibitor. Images of cells grown in the absence of inhibitor are shown in the top left (Control). Note that ES cell colonies are smaller and retain undifferentiated morphology after complete inhibition of SFK activity.

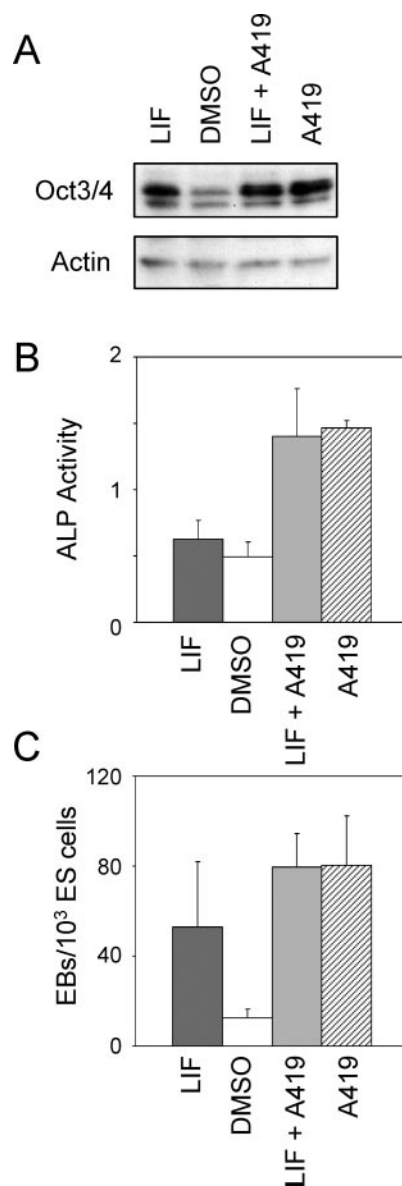


activity in vitro with an  $IC_{50}$  value of 20 nM but is much less potent for Lck, with an  $IC_{50}$  value of 6.88  $\mu$ M (Blake et al., 2000). Low concentrations of SU6656 (2  $\mu$ M) have been reported to induce differentiation of ES cells (Anneren et al., 2004), although the effect of this compound on overall SFK activity in ES cells was not investigated. Figure 4A shows that 2  $\mu$ M SU6656 produced only a partial block of SFK activity, and a complete block was not observed, even at the highest concentration tested (8  $\mu$ M). None of these concentrations supported LIF-independent colony formation, and higher concentrations were toxic to ES cells (data not shown). These data are consistent with the recent observation that Yes or other SU6656-sensitive kinases contribute to self-renewal (Anneren et al., 2004).

Together, these results strongly suggested that ES cell fate in response to inhibitor treatment (e.g., self-renewal versus differentiation) was dependent on the subset of SFKs that remained active at different inhibitor concentrations. Particularly interesting were the results obtained for SKI-1, which produced a clear dose-dependent inhibition of SFK activity in ES cells over the range of concentrations tested (Fig. 4A). To determine whether SFKs display differential sensitivity to SKI-1, we determined  $IC_{50}$  values for purified recombinant Fyn, Hck, Src, and Yes using an in vitro assay system (Table 2). It is interesting to note that the SKI-1  $IC_{50}$  values obtained for Fyn (193  $\mu$ M) and Src (80  $\mu$ M) were significantly higher than those observed for Hck (35  $\mu$ M) and Yes (21  $\mu$ M). This observation suggests that low concentrations of SKI-1 selectively inhibit Hck and Yes in ES cells while sparing Src and Fyn, which are clearly active both in cycling ES cells and embryoid bodies (Figs. 1 and 3). Because Hck and Yes have been implicated in self-renewal, selective inhibition of these kinases may be sufficient to induce differentiation despite the presence of LIF.

**Complete SFK Inhibition Prevents Differentiation of ES Cells While Maintaining Pluripotency.** The results presented in Fig. 4 suggested that complete inhibition of SFK activity allowed the persistence of undifferentiated ES cells, even in the absence of LIF. To determine whether SFK inhibitor-treated ES cells retained pluripotency, we measured their Oct3/4 protein levels, ALP activity, and ability to form EBs (Zandstra et al., 2000). ES cells were grown with and without LIF and 300 nM A-419259 for 5 days. A-419259 was chosen for these experiments because it was the most potent inhibitor of SFK activity tested, resulting in a complete block in SFK autophosphorylation at 300 nM (Fig. 4A). In addition, A-419259  $IC_{50}$  values for Fyn, Src, Hck, and Yes lie within a relatively narrow range, varying by only 2-fold for Src, Fyn, and Yes (Table 2). After inhibitor treatment, cells were pro-

cessed for immunoblotting or ALP assays, or inhibitor was removed and cells were trypsinized and plated for EB formation. As shown in Fig. 5A, LIF withdrawal led to a significant decrease in Oct3/4 levels in control cultures, consistent with the loss of pluripotency and the onset of differentiation. Remarkably, complete inhibition of SFK activity with A-419259 prevented this decrease, consistent with the persistence of undifferentiated colonies after LIF withdrawal from inhibitor-treated cultures. Similar results were observed for ALP activity (Fig. 5B) and EB formation (Fig. 5C); in both cases, inhibitor-treated cultures retained pluripotent cells despite 5 days in the absence of LIF. Similar results were observed after treatment of ES cells with PP2 (data not shown). These



**Fig. 5.** Src family kinase activity is required for ES cell differentiation. ES cells ( $10^4$ ) were plated in six-well tissue culture plates in ES medium in the presence of LIF. After 24 h, the cells were washed with PBS, and fresh medium was added containing LIF and the DMSO carrier solvent (LIF), DMSO in the absence of LIF (DMSO), LIF plus 0.3  $\mu$ M A-419259 (LIF+A419), or 0.3  $\mu$ M A-419259 alone (A419). All media were changed after 48 h of further growth. After 5 days, cultures were assayed for Oct3/4 expression (A), alkaline phosphatase activity (B), or EB formation (C) as described under *Materials and Methods*.

TABLE 2

Inhibition of the Src family kinases Fyn, Hck, Src, and Yes by SKI-1 and A-419259

Kinase activity was determined using recombinant purified kinases and a FRET-based assay as described in the text. The  $K_m$  value for ATP was determined for each kinase first, and subsequent  $IC_{50}$  values for each inhibitor were determined in the presence of an ATP concentration equal to twice the  $K_m$  value.

	ATP $K_m$	SKI-1 $IC_{50}$	A-419259 $IC_{50}$
	$\mu$ M	nM	
Fyn	6.4 $\pm$ 1.5	192.9 $\pm$ 1.1	12.8 $\pm$ 1.1
Hck	12.8 $\pm$ 2.8	35.3 $\pm$ 1.2	35.6 $\pm$ 1.1
Src	12.6 $\pm$ 0.8	79.9 $\pm$ 1.1	5.3 $\pm$ 1.0
Yes	8.0 $\pm$ 2.4	21.2 $\pm$ 1.1	5.3 $\pm$ 1.0

results strongly suggest that SFK activity is required for ES cells to exit self-renewal and undergo differentiation.

**SFK Activity Is Not Required for LIF-Induced Stat3 Activation.** Stat3 and Stat5 are tyrosine kinase-dependent transcription factors with demonstrated functions in the regulation of self-renewal and differentiation. Stat3 activation is both sufficient and necessary for self-renewal in murine ES cells (Niwa et al., 1998; Matsuda et al., 1999), whereas Stat5 has been implicated in the differentiation of hematopoietic cells (Buitenhuis et al., 2003; Hirokawa et al., 2003). We first looked for the LIF-dependent activation of these transcription factors in self-renewing ES cells. ES cells were grown for 24 h in the absence of LIF and then were challenged with LIF for 10 min. Stat3 and Stat5 were then immunoprecipitated and assayed for tyrosine phosphorylation by immunoblotting. As expected, Stat3 was rapidly phosphorylated after the addition of LIF (Fig. 6A). In contrast, we were unable to detect phosphorylation of Stat5 in either control or LIF-stimulated cells (Fig. 6A). Furthermore, we were unable to detect Stat5 phosphorylation in cells grown in the absence of LIF for up to 48 h (data not shown). These data suggest that Stat5 does not play a role in ES cell self-renewal or the early stages of differentiation.

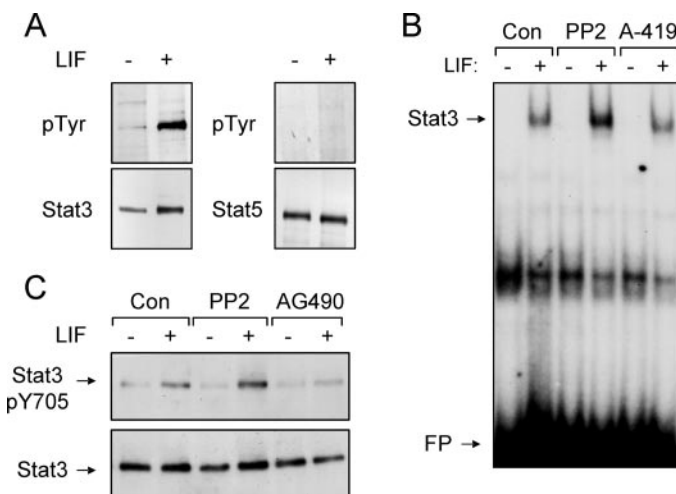
We next tested the effect of complete SFK inhibition on Stat3 activation. Previous results with the inhibitor SU6656 suggested that SFK activity was not required for Stat3 activation (Anneren et al., 2004). However, SU6656 produces an incomplete block in SFK activity at these concentrations (Fig. 4), leaving the contribution of SFKs to LIF-induced Stat3 activation an open question. To clarify this issue, ES cells were grown for 24 h in the absence of LIF and in the presence or absence of A-419259 (100 nM) or PP2 (10  $\mu$ M). Cells were

then challenged with LIF for 10 min, and Stat3 DNA-binding activity was assayed by EMSA using a radiolabeled oligonucleotide probe derived from a Stat3-binding sequence in the *c-fos* promoter (SIE). As shown in Fig. 6B, Stat3-SIE complexes were readily detected in LIF-treated cells, consistent with published reports (Boeuf et al., 1997; Niwa et al., 1998). Neither Src inhibitor suppressed the Stat3 signal, consistent with the undifferentiated phenotype of inhibitor-treated cells. PP2 also did not affect phosphorylation of Stat3 Tyr705 in response to LIF (Fig. 6C). In contrast, phosphorylation of this activating tyrosine was completely blocked by AG490, a Jak kinase inhibitor. These results show that SFK activity is not required for Stat3 activation by LIF in murine ES cells, despite the observation that Hck and other Src kinases contribute to Stat activation in other cellular contexts (Smithgall et al., 2000; Schreiner et al., 2002).

**SFK Activity Is Required for EB Development.** To address the role of SFK activity in the earliest stages of ES cell differentiation more directly, we tested the impact of the selective SFK inhibitor A-419259 on EB formation. EB cultures were started in the presence of either DMSO or 300 nM A-419259 and were allowed to develop for 6 to 12 days. After 6 days, EBs grew to 1 to 2 mm in size and showed spherical morphology as expected (Fig. 7A). In contrast, EBs grown in the presence of A-419259 were irregularly shaped and significantly smaller than those grown in the absence of inhibitor. It is interesting that after 12 days of growth, there was little morphological difference between the treated and untreated EBs, indicating that reversible SFK inhibition substantially delays but does not prevent EB formation.

The decreased growth rate of the inhibitor-treated EBs suggested that differentiation of these cells might also be delayed. To test whether inhibitor-treated EBs retained pluripotent cells, we performed secondary EB assays. In these experiments, EBs are dissociated into single cells and are replated under conditions for EB formation in semisolid medium. The number of secondary EBs that form after 10 days reflects the number of pluripotent cells remaining in the primary EB (Chan et al., 2003). Secondary EB assays were performed for EBs grown for 6 or 12 days. Previous reports have shown that a majority of pluripotent cells in a developing EB are lost within 6 days (Qu et al., 1997). As shown in Fig. 7B, this loss is clearly reflected in the relatively small number of secondary EBs formed by cells taken from control 6-day EBs formed in the absence of the inhibitor. In contrast, EBs treated with A-419259 for 6 days contained approximately 5-fold more cells able to form secondary EBs compared with untreated EBs. However, EBs treated with inhibitor for 12 days eventually lose their pluripotent cells and produce small numbers of secondary EBs, as observed for untreated cultures. These results suggest that SFK activity is required for efficient exit from the self-renewal program and the onset of differentiation.

To further define the time frame during which SFK activity is required for differentiation, EB development was initiated for 3, 4, or 5 days before the addition of the inhibitor. On day 6, all EBs were harvested and trypsinized to single cells, and equal cell numbers were replated in secondary EB assays. As shown in Fig. 7C, the longer the EBs were allowed to develop before the addition of the inhibitor, the less effect it had on EB formation. The effect of the inhibitor on EB development was greatly diminished by day 4, demonstrating



**Fig. 6.** Src kinase inhibitors do not affect LIF-induced activation of Stat3. A, ES cells were grown in the absence of LIF for 24 h and then were stimulated with LIF for 10 min. Stat3 and Stat5 were immunoprecipitated from ES cell lysates and were immunoblotted for tyrosine phosphorylation (pTyr) or for the Stat protein as indicated. B, ES cells were incubated overnight in the absence of LIF and in the absence (Con) or presence of the SFK inhibitors PP2 (10  $\mu$ M) or A-419259 (100 nM). Cells were then treated with LIF (+) or were left untreated (-) for 10 min, and nuclear extracts were prepared and tested for Stat3 DNA binding activity by EMSA using a radiolabeled SIE probe. The arrows indicate the positions of the Stat3-SIE complex (Stat3) and free probe (FP). C, ES cells were treated with PP2, the Jak kinase inhibitor AG490, or left untreated (Con) as described for B. Stat3 was immunoprecipitated from cell lysates and was probed with antibodies specific for the active form of Stat3 (pY705; top) or for Stat3 protein (bottom).



that SFK activity plays an essential role in the earliest stages of embryoid body formation.

To ensure that the delay in differentiation observed for A-419259-treated EBs correlated with a corresponding change in transcriptional regulation of differentiation markers, we used RT-PCR to test control and inhibitor-treated EBs and self-renewing ES cells for Hck and Gata4 transcript levels. As shown in Fig. 7D, control EB formation correlated with down-regulation of Hck and up-regulation of Gata4, consistent with the loss of pluripotent stem cells and the onset of differentiation. In contrast, A-419259-treated EB cultures continued to express Hck and failed to induce Gata4. These results also support a role for SFK activity early in EB development.

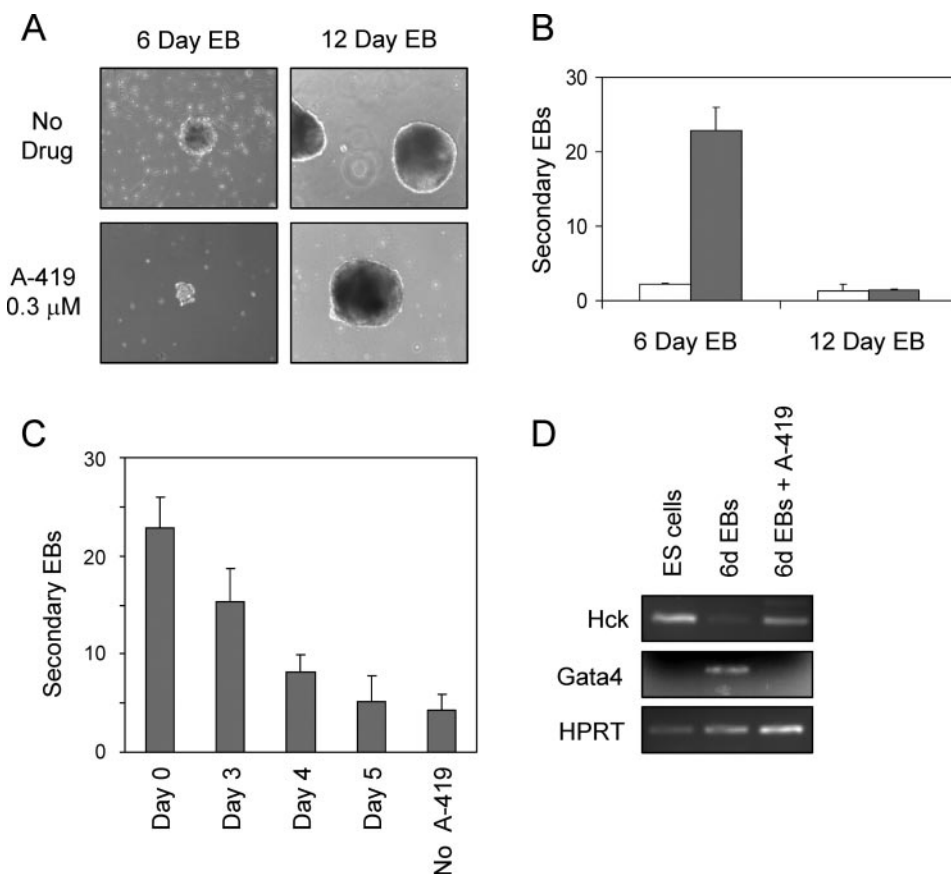
## Discussion

In this study, we show for the first time that SFK activity is required for initiation of the differentiation program after LIF withdrawal in murine ES cells. SFKs are known to regulate a wide variety of cellular functions including proliferation, adhesion, differentiation, and survival (Parsons and Parsons, 1997; Thomas and Brugge, 1997; Abram and Courtneidge, 2000). All of these processes are involved in early embryogenesis, and our surprising observation that seven of eight SFKs are expressed in ES cells suggests that this kinase family makes a substantial contribution to the network of tyrosine kinase signaling pathways in these cells. Three of the seven family members showed constitutive activity in self-renewing ES cells grown in the presence of LIF and serum (Hck, Src, and Fyn; Fig. 1B). Of these, Hck has

been implicated previously in LIF signaling in ES cells (Ernst et al., 1996, 1999), whereas Src and Fyn may be responsive to serum growth factors as observed in other cell types (Abram and Courtneidge, 2000). In addition, Fyn and other SFKs are key intermediates, coupling fibroblast growth factors to mesoderm induction in amphibian early embryogenesis (Weinstein et al., 1998; Weinstein and Hemmati-Brivanlou, 2001). Whether Fyn plays a similar role in mammalian ES cells will require further investigation.

One surprising result was the ES cell expression of SFKs normally associated with lineage-restricted expression patterns in the adult, including Hck and Fgr (myeloid leukocytes) as well as Lck (T lymphocytes). Distinct patterns of SFK gene expression were also observed as ES cells differentiated to embryoid bodies. Particularly striking were changes in the expression patterns for Hck and Lck (Fig. 2). Hck expression was down-regulated very early in EB formation with kinetics that followed the loss of pluripotency. Lck expression was also down-regulated, although more slowly and in parallel with the appearance of differentiation markers. These observations suggest that Hck and Lck contribute to self-renewal or the earliest stages of EB formation. In contrast, Src, Fyn, Lyn, and Yes expression did not change during EB formation, suggestive of roles in signaling responses to growth factors or cell-cell contact essential for differentiation.

To provide insight into the overall function of SFKs in the self-renewal and differentiation of ES cells, we used the SFK inhibitors SKI-1, PP2, A-419259, and SU6656. We were surprised to find that ES cells cultured with inhibitor concentrations sufficient to completely block SFK autophosphoryla-



**Fig. 7.** Src kinase activity is required for EB development. **A**, micrographs of 6- and 12-day EBs grown with or without 0.3  $\mu$ M A-419259. **B**, secondary EB formation from 6- and 12-day primary EBs grown with ( $\blacksquare$ ) or without ( $\square$ ) 0.3  $\mu$ M A-419259. **C**, SFK inhibitor treatment is required during the first 3 days to affect EB formation. All EBs were grown for a total of 6 days before dissociation and plating for the secondary EB assay. A-419259 (0.3  $\mu$ M) was added at the beginning of primary EB culture and 3, 4, or 5 days later. Untreated cells are included as a control (no A-419; far right). **D**, RT-PCR analysis of Hck, Gata4, and HPRT expression in ES cells versus 6-day EBs treated with or without A-419259 (A-419).

tion in vivo consistently grew as small, undifferentiated colonies, regardless of whether LIF was included in the culture medium (Fig. 4). Furthermore, ES cells treated with A-419259, the most potent of these inhibitors, expressed high levels of Oct3/4 and alkaline phosphatase activity and retained the ability to form EBs after inhibitor withdrawal, despite the absence of LIF (Fig. 5). In addition, this inhibitor substantially delayed EB formation, demonstrating a requirement for SFK activity in ES cell differentiation and early embryogenesis. EBs grown in the presence of A-419259 for 6 days formed new EBs upon dissociation and replating in the absence of the inhibitor (Fig. 7B). This inhibition of differentiation was transient, however. EBs treated for 12 days with A-419259 grew to a size equal to untreated EBs, and secondary EB assays showed that these 12-day inhibitor-treated EBs had differentiated to the same extent as untreated EBs (Fig. 7, A and B). These observations suggest that individual SFKs have essential signaling roles in the initiation of development and that inhibition of these pathways delays differentiation. In addition, SFK inhibition may cause growth suppression, slowing the rate of EB formation.

Inherent in any inhibitor study is the potential for nonspecific activity against alternative kinase targets. To control for this possibility, we tested multiple SFK inhibitors from different structural classes. In each case, the same results were obtained: partial inhibition of SFK kinase activity resulted in the loss of undifferentiated colony morphology, whereas complete suppression of SFK activity blocked differentiation while maintaining pluripotency (Fig. 4). These observations argue against a role for non-SFK targets. In addition, we demonstrated that direct inhibition of the PDGF receptor and c-Abl, two kinases known to cross-react with PP2, did not affect ES cell growth or differentiation (data not shown). Taken together, these data strongly implicate SFKs as the biologically relevant targets for these compounds.

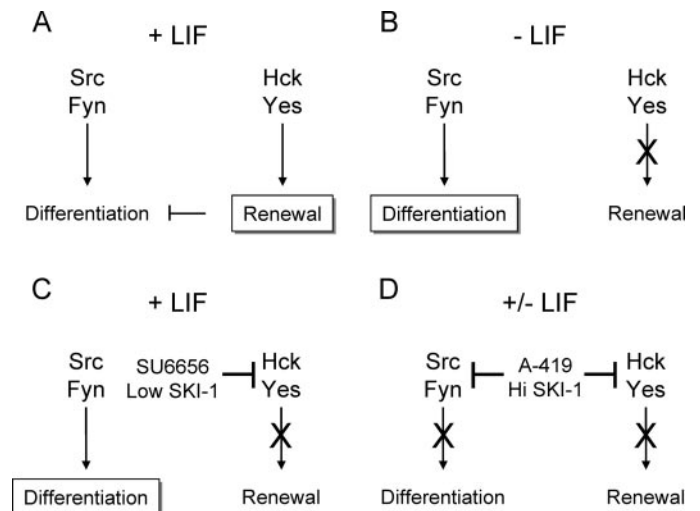
Together with previous studies, our inhibitor data suggest a model in which individual members of the Src kinase family independently control ES cell fate (Fig. 8). Some SFKs may contribute to Stat3-independent self-renewal in the presence of LIF (Fig. 8A). The rapid silencing of Hck transcription upon LIF withdrawal and EB formation (Fig. 2) and its connection to the LIF signaling pathway (Ernst et al., 1994, 1996, 1999), as well as recent work showing that suppression of Yes expression induces loss of pluripotency (Anneren et al., 2004), identify these two kinases as potential modulators of self-renewal. Other members of the Src kinase family may be required for efficient exit from the self-renewal pathway and differentiation after LIF withdrawal (Fig. 8B). Our observation that c-Src and Fyn are constitutively expressed and active in both cycling ES cells and embryoid bodies strongly suggests that these SFKs selectively contribute to these processes. Src and Fyn activity may simply drive the cell cycle as differentiation proceeds or may contribute directly to the differentiation program, as suggested by studies in *X. laevis* embryogenesis (Weinstein et al., 1998).

This model also accounts for the opposing actions of low and high concentrations of SFK inhibitors on ES cells in culture. For example, treatment of ES cells with low or intermediate doses of SKI-1 is likely to result in the inhibition of the renewal-related kinases Hck and Yes, whereas Fyn and Src remain active. As a consequence, ES cells enter the differentiation pathway, despite the presence of LIF (Fig.

8C). The IC<sub>50</sub> data obtained with SKI-1 supports this idea. Indeed, Yes is almost 10 times more sensitive to SKI-1 inhibition than to Fyn, whereas Hck is at least 5-fold more sensitive (Table 2). SU6656, which also causes ES cell differentiation in the presence of LIF, demonstrates similar selectivity for Yes relative to Fyn and Src in vitro (Blake et al., 2000), leading to the suggestion that Yes is its preferred target in ES cells (Anneren et al., 2004). Unlike Hck, however, constitutive Yes activity was not observed in self-renewing ES cells, and its expression does not change as a function of differentiation.

When higher concentrations of SKI-1 are used, or when ES cells are treated with the more potent inhibitors PP2 and A-419259, all SFK activity is blocked (Fig. 4A). In this case, SFK pathways for self-renewal and differentiation are both affected, and the net result is the appearance of small undifferentiated colonies (Fig. 4B) and a substantial delay in differentiation, as measured by EB formation (Fig. 7). These findings also imply that loss of pluripotency and the onset of differentiation may be linked to cell-cycle progression (Fig. 8D).

In summary, work presented here provides new evidence that embryonic stem cell fate can be manipulated through the use of small molecules, particularly those targeted to kinase signaling pathways (Ding et al., 2003; Ding and Schultz, 2004; Wu et al., 2004). A complete understanding of SFK signaling networks in ES cells has the potential to allow the manipulation of ES cell fate in vitro through the targeted



**Fig. 8.** Proposed model for the regulation of ES cell fate by the Src kinase family. A, data presented here and elsewhere strongly suggest that SFKs regulate at least two independent signaling pathways in ES cells. One pathway promotes self-renewal in the presence of LIF and is likely to include Hck and Yes. The second pathway functions to promote growth and differentiation and is likely to include Src and Fyn. When both pathways are active in cycling ES cells, the renewal pathway dominates, suppressing differentiation and allowing expansion of the pluripotent stem cell population. B, removal of LIF leads to the rapid loss of Hck expression and other signals for self-renewal, allowing initiation of the differentiation program. C, inhibition of renewal-related kinases such as Hck and Yes by SU6656 or by low concentrations of SKI-1 induces differentiation in the presence of LIF. This observation supports the idea that signals for differentiation are present in self-renewing ES cells but are repressed by SFK-induced signals for self-renewal independent of Stat3. D, inhibition of SFKs involved in both the renewal and differentiation pathways renders ES cells unable to differentiate in response to LIF withdrawal. These cells proliferate slowly and retain the characteristics of pluripotent cells, even in the absence of LIF.

inhibition of individual kinases. The similar structures of tyrosine kinases in general and Src family kinases in particular present a challenge to the design of SFK isoform-selective inhibitors, which will be essential for ultimately understanding the contribution of each Src family member to ES cell growth and differentiation.

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