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

Malcolm A. Meyn III, Steven J. Schreiner, Teodora Pene Dumitrescu, Gerard J. Nau, and Thomas E. Smithgall

From the Departments of Molecular Genetics and Biochemistry (M.A.M., S.J.S., T.P.D., G.J.N., T.E.S), and Medicine (G.J.N.), University of Pittsburgh School of Medicine, E1240 Biomedical Science Tower, Pittsburgh, PA 15261

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Running title: Src family kinases and ES cell differentiation

Corresponding Author:

Thomas E. Smithgall, Ph.D.
Department of Molecular Genetics and Biochemistry
University of Pittsburgh School of Medicine
E1240 Biomedical Science Tower
Pittsburgh, PA 15261

Phone: (412) 648-9495
Fax: (412) 624-1401
tsmithga@pitt.edu

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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 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 with shared structural architecture. In this work, 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 as well as loss of the corresponding kinase proteins. 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 PP2 or SKI-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, blocked differentiation in response to LIF withdrawal. Surprisingly, 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.

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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 (Williams et al., 1988; Smith 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 (Hirano et al., 2000; Smithgall et al., 2000; Burdon et al., 1999). Kinase activation induces phosphorylation of the Stat3 transcription factor downstream, which is both necessary and sufficient for self-renewal (Niwa et al., 1998; Matsuda 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. established a correlation between the ability of truncated gp130 mutants to activate Hck and suppress ES cell differentiation (Ernst et al., 1999). Mutant gp130 also failed to activate Stat3, suggesting a connection between Hck and Stat3 activation in ES cells. This possibility is supported by the direct activation of Stat3 by active mutants of Hck and Src in transformed rodent fibroblasts (Schreiner et al., 2002; Yu et al., 1995). 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 anti-sense approach also led to differentiation, implicating Yes as the target for SU6656. On the other hand, work in *Xenopus* embryos has demonstrated a

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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). *Xenopus* Fyn and the unique Src homolog Laloo appear to be key intermediates linking FGF 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 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, while transcript levels of Src, Fyn, Yes and Lyn remained constant. Pharmacological inhibition of overall SFK activity with the highly potent antagonist A-419259 suppressed ES cell differentiation to embryoid bodies while preserving pluripotency, despite the absence of LIF. Furthermore, this inhibitor did not impact 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 SKI-1 also blocked differentiation while maintaining pluripotency in a manner similar to A-419259. Strikingly, 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.

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Materials and Methods

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

Immunoprecipitation (IP) and Immunoblot (IB) Analysis. Antibodies, applications and sources include: Src phosphospecific for IB (pY418), Biosource International KHO0171; c-Src for IP, Santa Cruz Biotechnology (SCB) sc-8056; c-Src for IB, SCB sc-18; Fyn for IP, BD Biosciences (BDB) 610163; Fyn for IB, SCB sc-16; Hck for IP, SCB sc-1428; Hck for IB, SCB sc-72; Lck for IP, SCB sc-433; Lck for IB, SCB sc-13; Lyn for IP, SCB sc-7274; Lyn for IB, SCB sc-15; Yes for IP, SCB sc-8403; Yes for IB, SCB sc-14; Fgr for IP, Serotec AHP657; Fgr for IB, SCB sc-17; Oct3/4 for IB, BDB 611202; Stat5 for IP and IB, SCB sc-835; anti-phosphotyrosine PY99 for IB, SCB sc-7020; actin for IB, Chemicon MAB1501.

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-PAGE loading buffer (0.5 ml). For immunoprecipitation experiments, cells ($5x10^6$) were washed and re-suspended in 1 ml RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with 1 mM Na_3VO_4, 1 mM NaF, 0.25 µl Benzonase (Novagen) and a protease inhibitor cocktail (Calbiochem). For protein-tyrosine phosphatase treatment, immunoprecipitates were washed with phosphatase buffer (25 mM MOPS pH 7.0, 50 mM NaCl, 1 mM DTT, 0.05% Tween-20) and treated with 25 U *Yersinia enterocolitica* protein tyrosine phosphatase (Calbiochem) prior to immunoblot analysis.

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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). Clones for expression of murine Fgr, Lyn, and Src were obtained from the ATCC and subcloned into the same vector. Culture and transient transfection of human 293T cells was performed as described elsewhere (Rogers et al., 1996).

SFK Inhibitors. SU6656, Src Kinase Inhibitor-1 (SKI-1), and PP2 were purchased from Calbiochem. A-419259 was provided by Abbott Bioresearch, Worcester, MA (Wilson et al., 2002).

Electrophoretic Mobility Shift Assay (EMSA) for Stat3. The EMSA for Stat3 activation has been described elsewhere (Schreiner et al., 2002; Choi and Smithgall, 2004). Briefly, ES cells were centrifuged, washed once with PBS, and nuclear extracts were made using the Nuclear Extract Kit (Active Motif). The probe used for the Stat3 EMSA is based on the *sis*-inducible element (SIE). Complementary SIE oligonucleotides (20 pmol) were annealed in 10 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and labeled with [α-^{32}P] dATP and the Klenow fragment of DNA polymerase. EMSA was performed with 10 µg of ES cell nuclear extract and 40,000 cpm of labeled SIE probe in a final volume of 20 µl. 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). Poly dT-primed cDNA was synthesized from 2 µg of total RNA using Moloney murine leukemia virus reverse transcriptase. For semi-quantitative analysis, one-fifteenth of each RT reaction was then

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used in 50 µl 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 µl) of each reaction were run on 2% agarose gels and 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 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 GAPDH as the internal control. Changes in the expression of each gene were calculated from cycle counts (CT) where gene expression = $2^{\Delta\Delta C}{}_{T.}$ 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, Inc. Murine Hck was expressed in Sf9 insect cells as a an N-terminal hexahistidine fusion protein and purified as described (Schindler et al., 1999). Kinase assays were performed using the FRET-based Z'-Lyte Src kinase assay kit according to the manufacturer's instructions (Invitrogen). All assays were done in triplicate in low volume, non-binding 384-well plates (Corning) and read on a Gemini XS microplate spectrofluorometer (Molecular Devices). The assay was first optimized for linearity with respect to incubation time and kinase concentration. The K_m for ATP was then determined for each kinase over an ATP concentration range of 0 to 200 µM with a saturating concentration of substrate peptide (2 µM). The K_m was determined by non-linear regression curve fitting using the Michaelis-Menton equation and Prism software (GraphPad). IC_{50} values were determined for A-419259 and SKI-1 in the presence of an ATP concentration equal to twice the K_m for each kinase. IC_{50} values were calculated using a four-parameter sigmoidal curve fit and Prism software.

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Alkaline Phosphatase (ALP) Assay. ALP assays were conducted as described (Zandstra et al., 2000). Cells were counted prior to lysis and results expressed as units ALP activity/1000 cells.

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

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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). While the individual Src family kinases Hck and Yes have been studied in the context of ES cell growth and differentiation (Ernst et al., 1994; Ernst et al., 1996; Ernst et al., 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 utilized 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 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 (Figure 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 due to 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 (Figure 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

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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 (Wilson et al., 2002; Schreiner 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 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) prior to lysis or incubation of the precipitated kinases with a protein-tyrosine phosphatase prior to immunoblotting both resulted in a substantial reduction in the pY418 signal, confirming specificity for the active form of each kinase (data not shown).

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Src family kinase expression profiles in developing embryoid bodies. 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).

As outlined in the Introduction, previous reports have suggested a role for SFKs in the regulation of *Xenopus* 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 extra-embryonic 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 developing EBs. Expression of Lyn, Fyn, Src, and Yes remained constant throughout the twelve 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

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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 transcript still detectable in 12 day EBs.

We next investigated the decline in Hck and Lck expression with greater resolution through the use of quantitative real-time RT-PCR. Remarkably, Hck demonstrated a 30-fold decline in transcript levels during the initial 3 days of EB formation, while Lck displayed a slower rate of decline (Figure 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 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, 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

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markedly as a function of EB formation, while Fgr declined slightly, consistent with the RT-PCR result. Interestingly, Lyn levels also declined 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 either in 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 towards 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 μM 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 μM and nearly complete inhibition at 20 μM. 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.

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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 cell 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 μM) dispersed and appeared to differentiate despite the presence of LIF. At 20 μM 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 following treatment with 5 μM PP2, with nearly complete inhibition at 10 μM. As observed with SKI-1, partial inhibition of SFK activity with PP2 also promoted loss of undifferentiated colony morphology in the presence of LIF, while complete inhibition correlated with persistence of small undifferentiated colonies in the presence or absence of LIF. A potential complication of studies utilizing PP2, however, is non-specific inhibition of other protein-tyrosine kinases, including the PDGF receptor, c-Abl, and c-Kit (Tatton et al., 2003; Blake et al., 2000). To ensure that the phenotype observed with PP2 was not a result of inhibition of these kinases, we treated ES cells with either the PDGFR inhibitor AG1295 or the c-Abl and c-Kit inhibitor STI-571 (imatinib) in the presence or absence of LIF. Neither of these inhibitors affected undifferentiated ES cell colony formation, suggesting that the observed effects of PP2 treatment were due to SFK inhibition (data not shown).

A-419259 displays very high potency towards SFKs both in vitro and in vivo and unlike PP2 is 1000-fold less potent towards 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, striking similar

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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 activity in vitro with an IC_{50} of 20 nM but is much less potent for Lck, with an IC_{50} of 6.88 μM (Blake et al., 2000). Low concentrations of SU6656 (2 μM) 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. Fig. 4A shows that 2 μM SU6656 produced only a partial block of SFK activity, and a complete block was not observed even at the highest concentration tested (8 μM). 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 vs. differentiation) was dependent upon 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). Interestingly, the SKI-1 IC_{50} values obtained for Fyn (193 μM) and Src (80 μM) were significantly higher than those observed for Hck (35 μM) and Yes (21 μM). 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

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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 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, alkaline phosphatase (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 five 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 two-fold for Src, Fyn and Yes (Table 2). Following inhibitor treatment, cells were processed 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 following 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 following treatment of ES cells with PP2 (data not shown). These 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

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self-renewal and differentiation. Stat3 activation is both sufficient and necessary for self-renewal in murine ES cells (Matsuda et al., 1999; Niwa et al., 1998), while 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 hours in the absence of LIF and then challenged with LIF for 10 minutes. Stat3 and Stat5 were then immunoprecipitated and assayed for tyrosine phosphorylation by immunoblotting. As expected, Stat3 was rapidly phosphorylated following 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 up to 48 hours (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 hours in the absence of LIF and in the presence or absence of A-419259 (100 nM) or PP2 (10 μM). Cells were then challenged with LIF for 10 minutes, and Stat3 DNA-binding activity was assayed by EMSA using a radio-labeled oligonucleotide probe based on 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 (Niwa et al., 1998; Boeuf et al., 1997). Neither Src inhibitor suppressed the Stat3 signal, consistent with the undifferentiated phenotype of inhibitor-treated cells. PP2 also did not affect phosphorylation of Stat3 Tyr 705 in response to

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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 (Schreiner et al., 2002; Smithgall et al., 2000).

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 allowed to develop for 6 to 12 days. After 6 days, EBs grew to 1-2 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. Interestingly, after twelve 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 replated under conditions for EB formation in semi-solid medium. The number of secondary EBs that form after ten 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

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approximately five-fold more cells able to form secondary EBs as compared to 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 prior to addition of the inhibitor. On day 6 all EBs were harvested, trypsinized to single cells, and equal cell numbers were re-plated in secondary EB assays. As shown in Fig. 7C, the longer the EBs were allowed to develop before the addition of inhibitor, the less effect it had on EB formation. The effect of the inhibitor on EB development was greatly diminished by day 4, demonstrating 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 as well as 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.

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Discussion

In this study we show for the first time that SFK activity is required for initiation of the differentiation program following LIF withdrawal in murine ES cells. SFKs are known to regulate a wide variety of cellular functions including proliferation, adhesion, differentiation, and survival (Abram and Courtneidge, 2000; Thomas and Brugge, 1997; Parsons and Parsons, 1997). All of these processes are involved in early embryogenesis, and our surprising observation that seven out 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 previously implicated in LIF signaling in ES cells (Ernst et al., 1999; Ernst et al., 1996), while 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 FGFs to mesoderm induction in amphibian early embryogenesis (Weinstein and Hemmati-Brivanlou, 2001; Weinstein et al., 1998). 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 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

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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 utilized the SFK inhibitors SKI-1, PP2, A-419259, and SU6656. Surprisingly, ES cells cultured with inhibitor concentrations sufficient to completely block SFK autophosphorylation *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 following 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 re-plating 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. 7A,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 non-specific 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 loss of undifferentiated colony morphology, while complete suppression of

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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., 1999; Ernst et al., 1996; Ernst et al., 1994), 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 following 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 contribute directly to the differentiation program as suggested by studies in *Xenopus* 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 inhibition of the renewal-related kinases Hck and Yes, while Fyn and Src remain active. As a consequence, ES cells enter the differentiation pathway despite the presence of LIF (Fig. 8C). The IC_{50} data obtained with SKI-1 supports this idea. Indeed, Yes is almost ten times more sensitive to SKI-1 inhibition than Fyn, while is Hck is at least five-

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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 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 and Schultz, 2004; Ding et al., 2003; Wu et al., 2004). A complete understanding of SFK signaling networks in ES cells has the potential to allow manipulation of ES cell fate in vitro through the targeted inhibition of individual kinases. The similar structures of tyrosine kinases in general and Src family kinases in particular presents 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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Footnote

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Figure Legends

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 stained with ethidium bromide (right panel). To control for contaminating genomic DNA (*gDNA*), RT-PCR reactions were repeated with the HPRT primer set in the presence or absence of reverse transcriptase (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 phosphate prior to 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 immunoprecipated and immunoblotted for pY418 and each kinase protein as in (*B*).

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

embryoid bodies (EBs). Murine GAPDH was used as an internal reference and self-renewing ES cells were used as the calibrator. Data are expressed as $\log_2$ fold changes in expression levels (see Materials and Methods).

Fig. 3. SFK protein levels and activity in developing embryoid bodies (EBs). (*A*) Hck and Lck were immunoprecipated from aliquots of ES cell and 6 day EB lysates and 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.

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 (5×10^5) were plated on 60 mm plates and grown in the presence of LIF for 24 hours. 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 upper left panels (Control). Note that ES cell colonies are smaller and retain undifferentiated morphology following complete inhibition of SFK activity.

Fig. 5. Src family kinase activity is required for ES cell differentiation. ES cells (10^4) were plated in 6-well tissue culture plates in ES medium in the presence of LIF. After 24 hours, the cells were washed with PBS and fresh medium was added containing LIF and the DMSO carrier

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solvent (LIF), DMSO in the absence of LIF (DMSO), LIF plus 0.3 μM A-419259 (LIF+A419), or with 0.3 μM A-419259 alone (A419). All medium was changed after 48 hours 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 Experimental Procedures.

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 hours and then stimulated with LIF for 10 min. Stat3 and Stat5 were immunoprecipitated from ES cell lysates and 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 μM) or A-419259 (100 nM). Cells were then treated with LIF (+) or 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 positions of the Stat3-SIE complex (Stat3) and free probe (FP) are indicated by the arrows. (C) ES cells were treated with PP2, the Jak kinase inhibitor AG490, or left untreated (Con) as described for part B. Stat3 was immunoprecipitated from cell lysates and probed with antibodies specific for the active form of Stat3 (pY705; upper panel) or for Stat3 protein (lower panel).

Fig. 7. Src kinase activity is required for embryoid body (EB) development. (*A*) Micrographs of 6 and 12 day EBs grown with or without 0.3 μM A-419259. (*B*) Secondary EB formation from 6 and 12 day primary EBs grown with (filled bars) or without (open bars) 0.3 μM 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 prior to dissociation and plating for the secondary EB assay. A-419259 (0.3 μM) was added at the beginning of primary EB culture and 3, 4, or 5 days

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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 vs. 6 day EBs treated with or without A-419259 (A-419).

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.

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TABLE 1

PCR primers used in this study

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

	PCR Primer Sequences (5'-3')		PCR product size (bp)	
Gene	Forward	Reverse	gDNA	cDNA
Hck	gactttgacccccagcacggagac	cacacttctccaaactgccca	679	274
Fgr	ggtacttcggaaagatcagta	cgcactaggtcctgtatggag	407	240
Lyn	gaacaccctggatgctgttt	tgaacctgagtcactcggc	123	123
Yes	ggattatgccacaaagttaa	gacaatataaatgggctctt	472	306
Fyn	ggtactttggaaaacttggc	gtaccagttgctgaagtgtt	1515	239
Lck	cctgacgactggccaagaa	gccaccgttgtctaggtta	756	264
Blk	cttttccctgtccgtgaaag	catccagacttcgccaaact	623	294
Src	cgcttcaggcatggcctatgt	gtggctcagtggacgtaaa	808	466
AFP	tcgtattccaacaggagg	aggcttttgcttcaccag	1226	173
Gata4	gcctgtatgtaatgcctgcg	ccgagcaggaatttgaagagg	3363	499
HPRT	ctcgaagtgttggatacagg	tggcctataggctcatagtg	1187	349

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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.

	ATP K_m (μM)	SKI-1 IC_{50} (nM)	A-419259 IC_{50} (nM)
Fyn	6.4 ± 1.5	192.9 ± 1.1	12.8 ± 1.1
Hck	12.8 ± 2.8	35.3 ± 1.2	35.6 ± 1.1
Src	12.6 ± 0.8	79.9 ± 1.1	5.3 ± 1.0
Yes	8.0 ± 2.4	21.2 ± 1.1	5.3 ± 1.0

Fig. 1

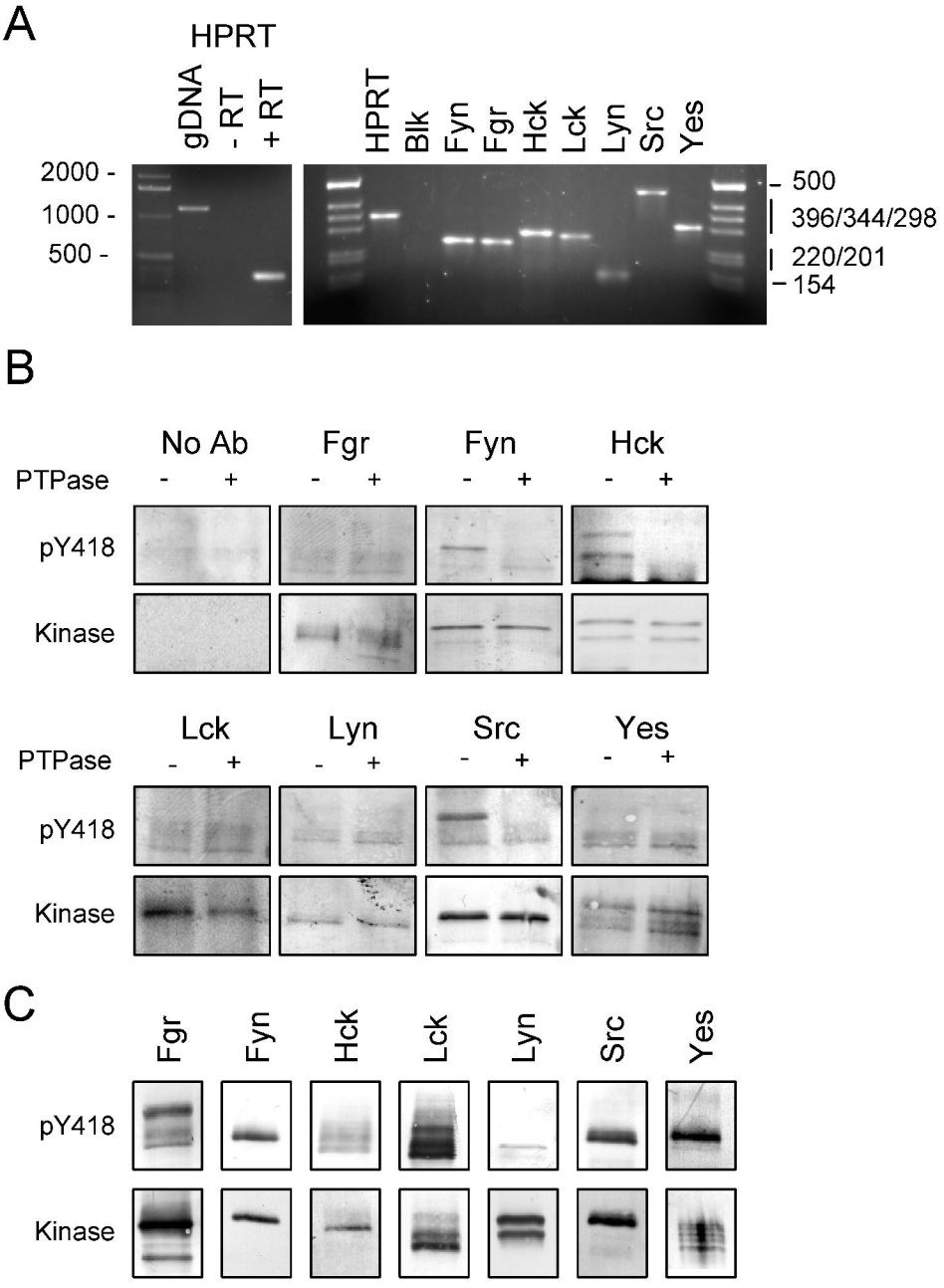

A
HPRT
gDNA
- RT
+ RT
2000 -
1000 -
500 -
HPRT
Blk
Fyn
Fgr
Hck
Lck
Lyn
Src
Yes
500
396/344/298
220/201
154
B
No Ab
Fgr
Fyn
Hck
PTPase
- + - + - + - +
pY418
Kinase
Lck
Lyn
Src
Yes
PTPase
- + - + - + - +
pY418
Kinase
C
Fgr
Fyn
Hck
Lck
Lyn
Src
Yes
pY418
Kinase

Fig. 2

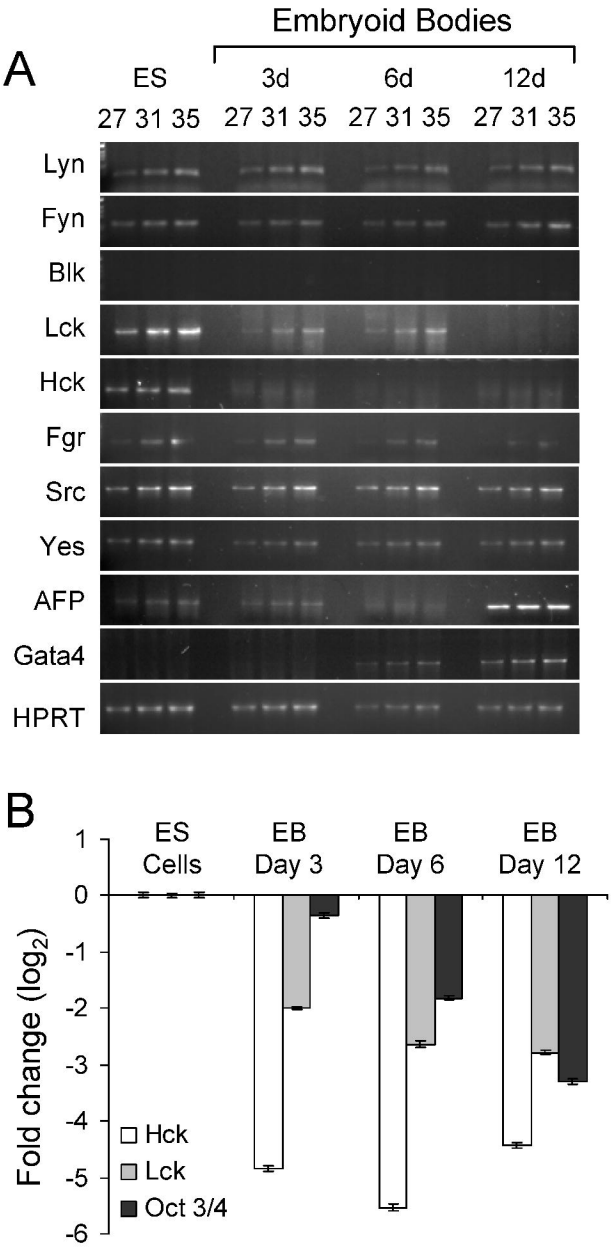

A
Embryoid Bodies
ES
3d
6d
12d
27 31 35 27 31 35 27 31 35 27 31 35
Lyn
Fyn
Blk
Lck
Hck
Fgr
Src
Yes
AFP
Gata4
HPRT
B
ES Cells
EB Day 3
EB Day 6
EB Day 12
Fold change (log2)
1
0
-1
-2
-3
-4
-5
-6
Hck
Lck
Oct 3/4

Fig. 3

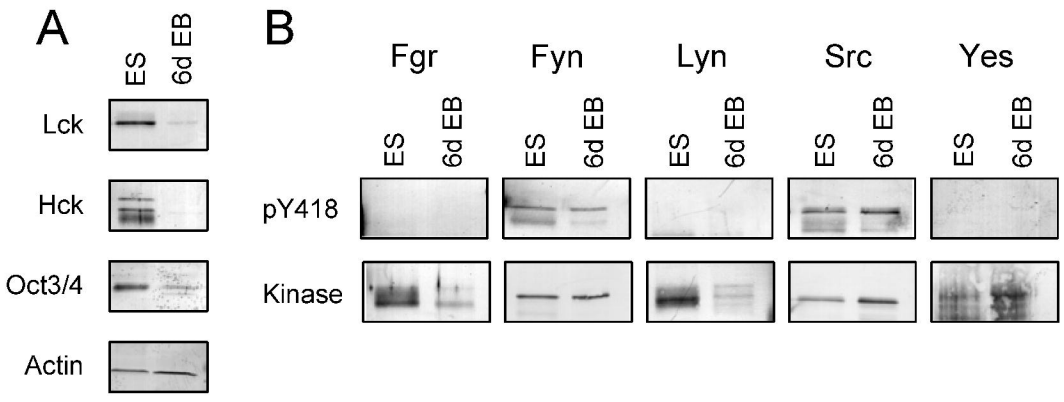
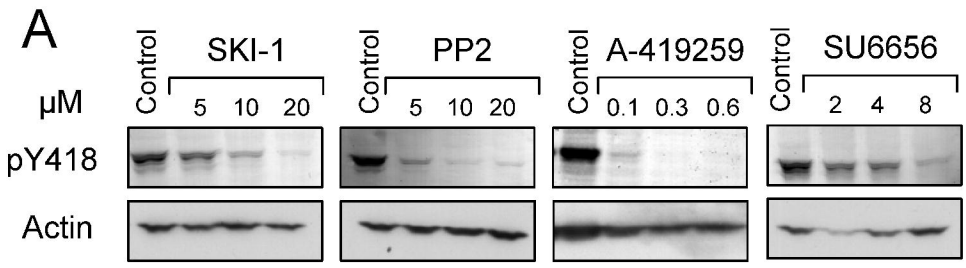

A
ES
6d EB
Lck
Hck
Oct3/4
Actin
B
Fgr
Fyn
Lyn
Src
Yes
ES
6d EB
pY418
Kinase

Fig. 4


A
Control
SKI-1
Control
PP2
Control
A-419259
Control
SU6656
μM
5 10 20
5 10 20
0.1 0.3 0.6
2 4 8
pY418
Actin

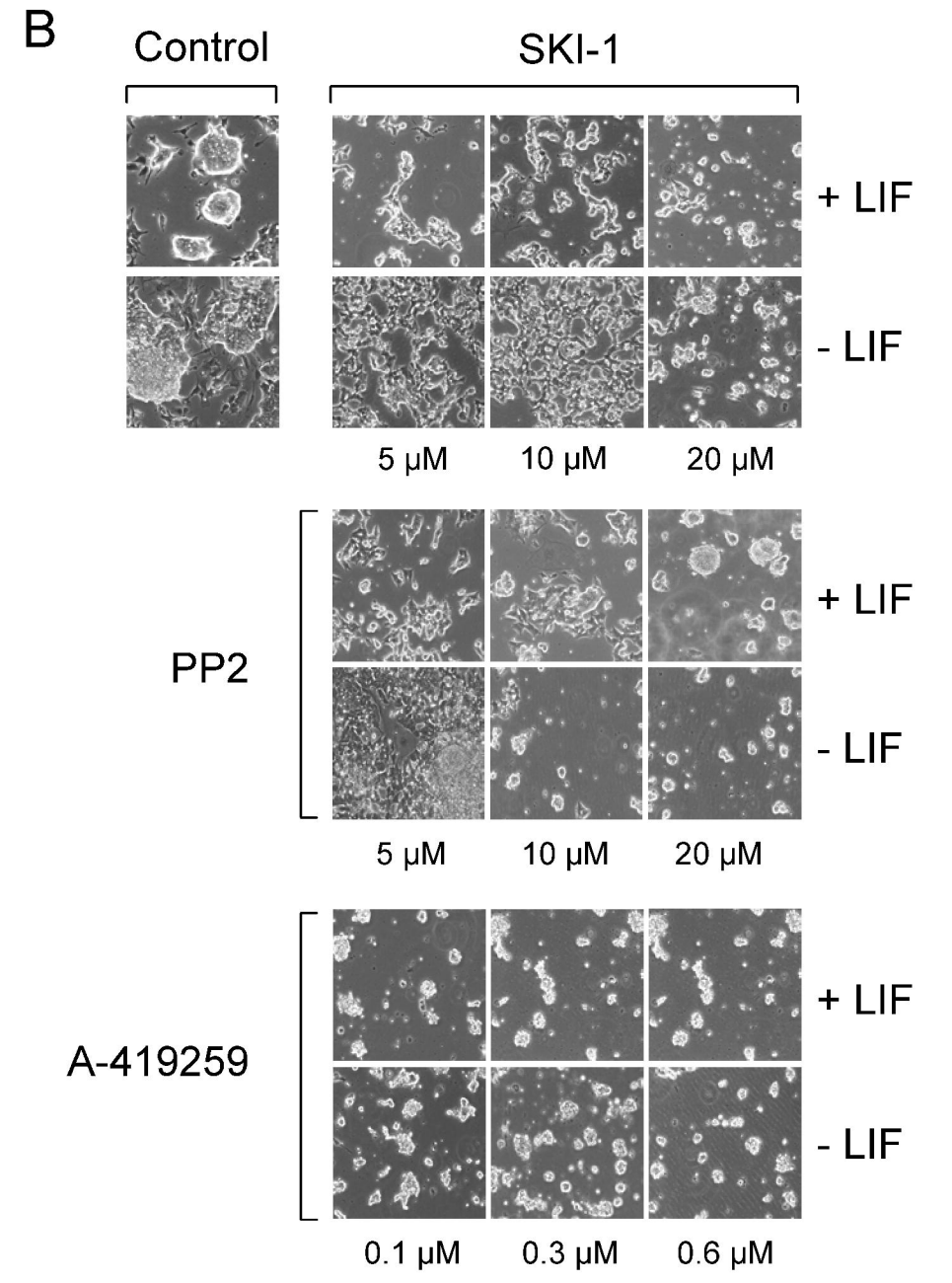

B
Control
SKI-1
+ LIF
- LIF
5 μM
10 μM
20 μM
PP2
+ LIF
- LIF
5 μM
10 μM
20 μM
A-419259
+ LIF
- LIF
0.1 μM
0.3 μM
0.6 μM

Fig. 5

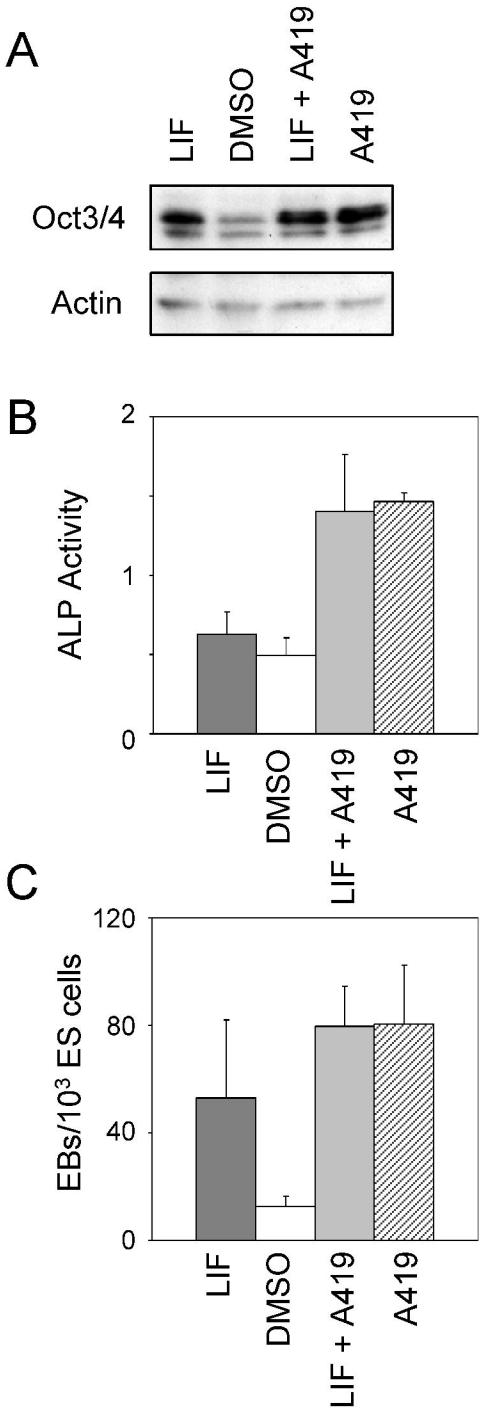

A
LIF
DMSO
LIF + A419
A419
Oct3/4
Actin
B
ALP Activity
2
1
0
LIF
DMSO
LIF + A419
A419
C
EBs/10^3 ES cells
120
80
40
0
LIF
DMSO
LIF + A419
A419

Fig. 6

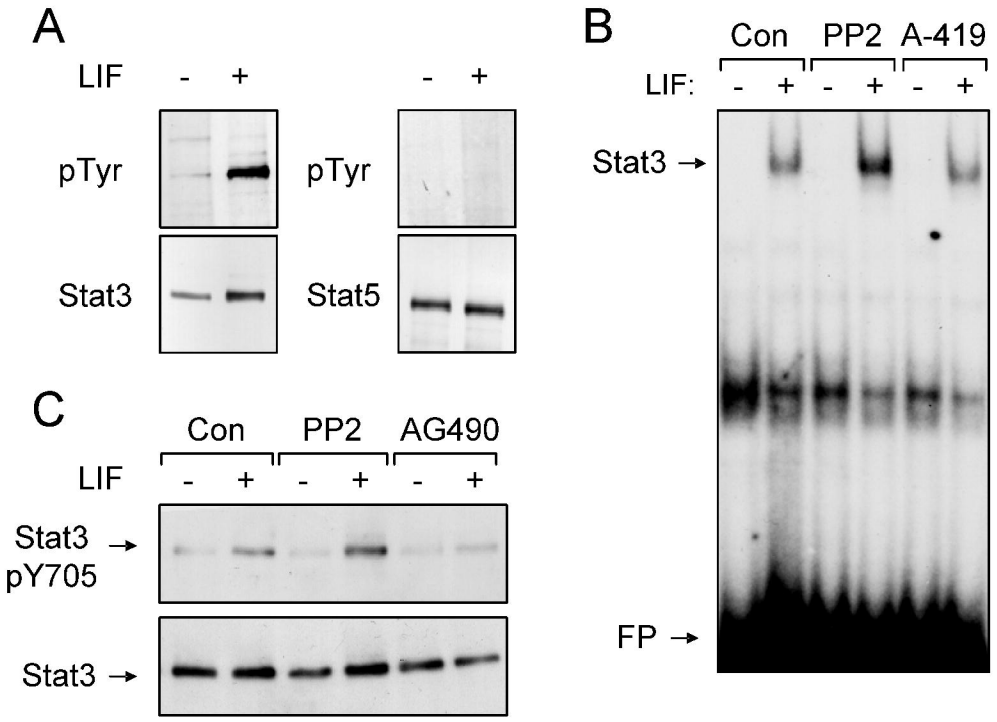

A
LIF
- +
- +
pTyr
pTyr
Stat3
Stat5
B
Con
PP2
A-419
LIF:
- + - + - +
Stat3
FP
C
Con
PP2
AG490
LIF
- + - + - +
Stat3
pY705
Stat3

Fig. 7

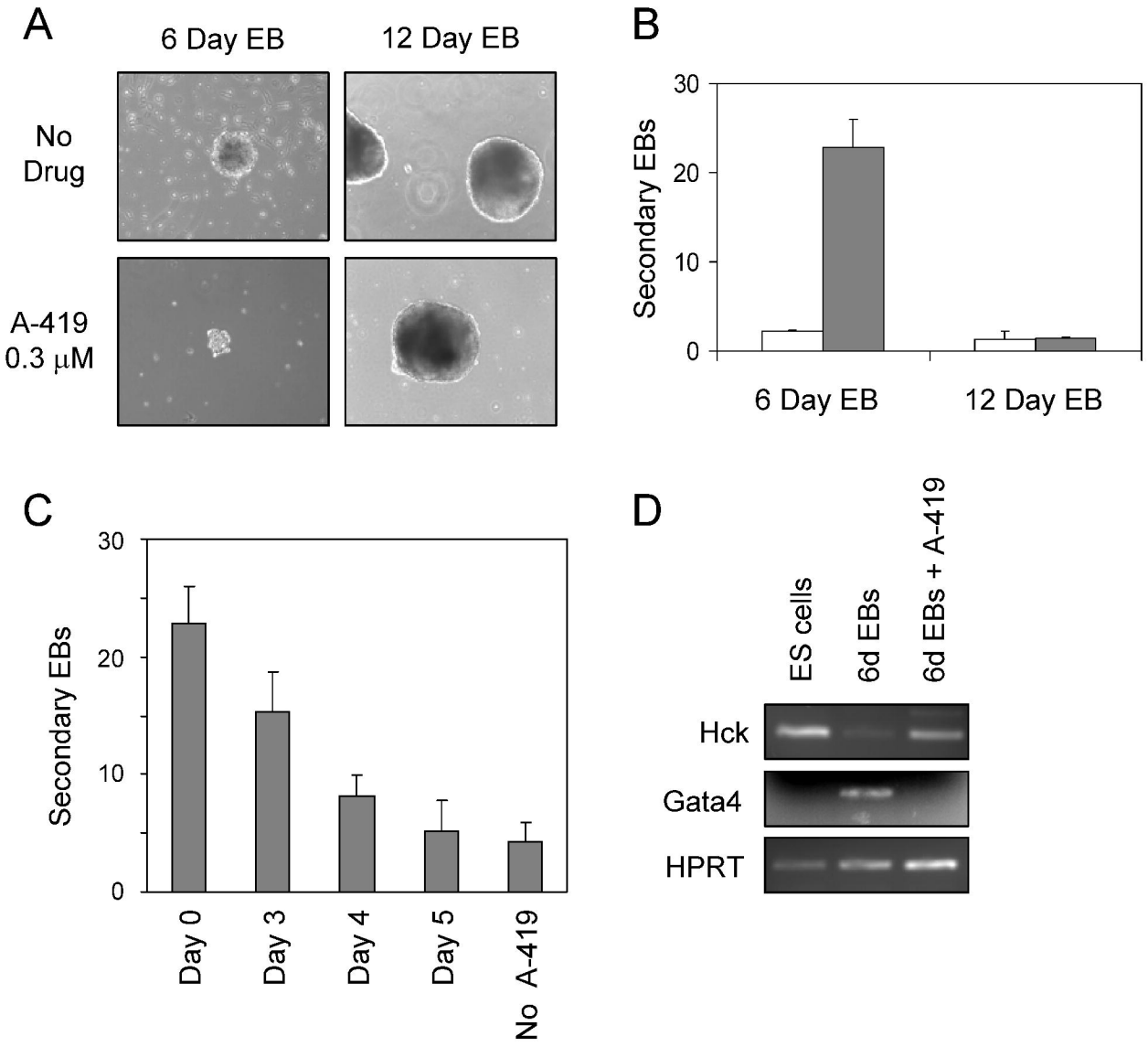
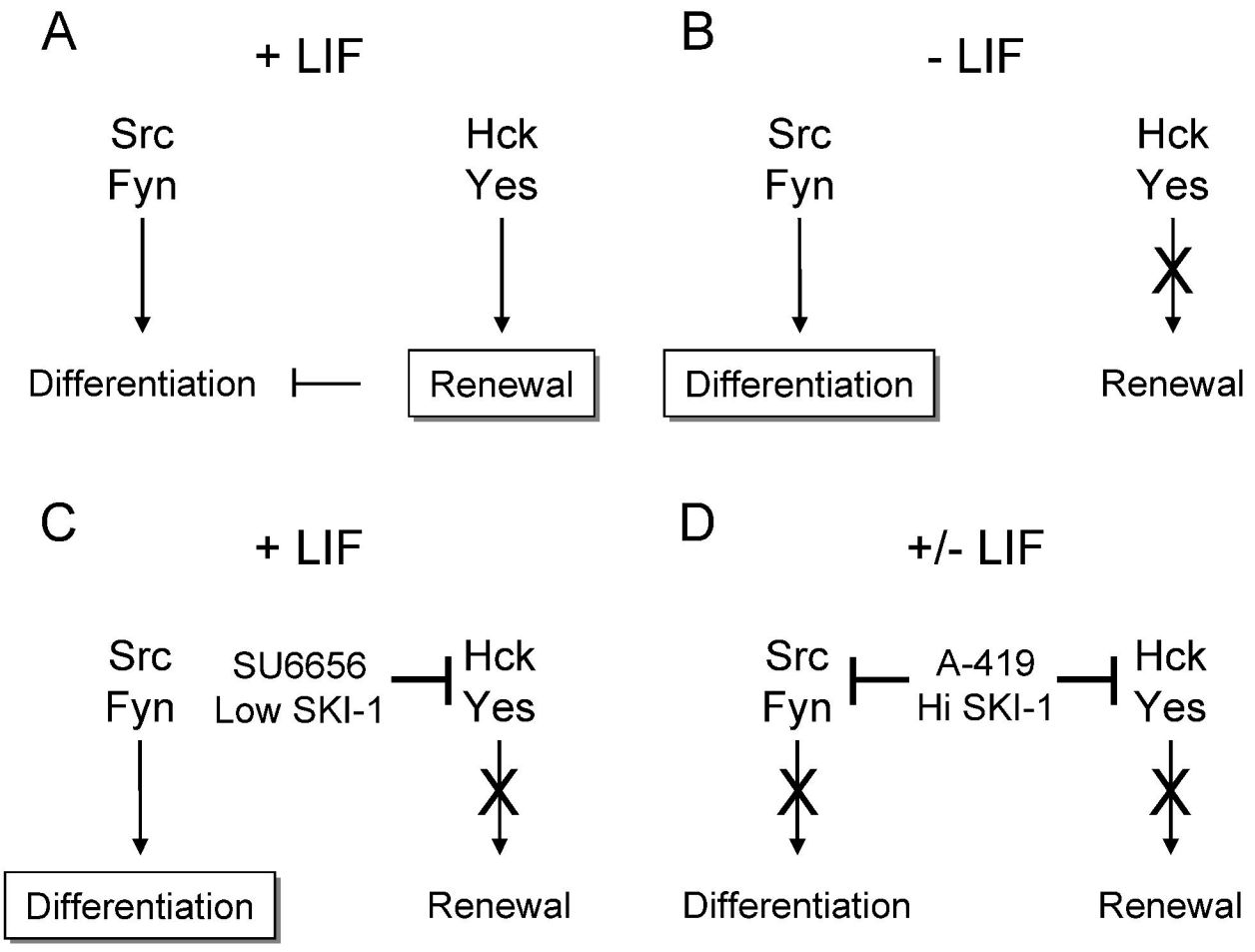

A
6 Day EB
12 Day EB
No Drug
A-419 0.3 μM
B
Secondary EBs
30
20
10
0
6 Day EB
12 Day EB
C
Secondary EBs
30
20
10
0
Day 0
Day 3
Day 4
Day 5
No A-419
D
ES cells
6d EBs
6d EBs + A-419
Hck
Gata4
HPRT

Fig. 8


A
+ LIF
Src
Fyn
Hck
Yes
Differentiation
Renewal
B
- LIF
Src
Fyn
Hck
Yes
Differentiation
Renewal
C
+ LIF
Src
Fyn
SU6656
Low SKI-1
Hck
Yes
Differentiation
Renewal
D
+/- LIF
Src
Fyn
A-419
Hi SKI-1
Hck
Yes
Differentiation
Renewal