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Chemical Genetics Identifies c-Src as an Activator of Primitive Ectoderm Formation in Murine Embryonic Stem Cells[#]

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Abstract

Multiple Src family kinases (SFKs) are present in murine embryonic stem (mES) cells. Whereas complete inhibition of all SFK activity blocks mES cell differentiation, inhibition of only c-Yes induces differentiation. Thus, individual SFKs may have opposing roles in the regulation of mES cell fate. To test this possibility, we generated SFK mutants with engineered resistance to a nonselective SFK inhibitor. The presence of an inhibitor-resistant c-Src mutant, but not analogous mutants of Hck, Lck, c-Yes, or Fyn, reversed the differentiation block associated with inhibitor treatment, resulting in the formation of cells with properties of primitive ectoderm. These results show that distinct SFK signaling pathways regulate mES cell fate and demonstrate that the formation of primitive ectoderm is regulated by the activity of c-Src.

INTRODUCTION

Embryonic stem (ES) cells are pluripotent, self-renewing cells derived from the inner cell mass of blastocysts (1,2). Maintenance of ES cell pluripotency and the initiation of differentiation pathways are regulated by signaling molecules, which are organized into complex signaling networks that transmit signals to the nucleus (3). In metazoans, transduction of extracellular signals to the interior of the cell often utilizes protein-tyrosine kinases and, accordingly, a number of tyrosine kinases have been implicated in the maintenance of ES cell pluripotency and the regulation of differentiation. These include kinases of both the receptor [fibroblast growth factor receptor 1 (FGFR1), epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptor (PDGFR)] and nonreceptor [Src family kinases (SFKs) and Janus kinases (Jak)] classes (4).

In humans, there are eleven SFKs, which regulate diverse cellular processes, including proliferation, cellular adhesion, differentiation, and survival (5,6). At least seven SFK isoforms are present in murine ES (mES) cells (7), but the function of SFKs in ES cells is unclear with some evidence supporting a role in self-renewal and some evidence supporting a role in differentiation.

In the absence of a feeder layer of mouse fibroblasts, cultured mouse ES cells require Leukemia Inhibitory Factor (LIF) to maintain pluripotency (8). Complete suppression of SFK activity with small molecule inhibitors blocks mES cell differentiation triggered by removal of LIF, supporting a role for SFKs in activation of mES cell differentiation (7). A role for SFK activity

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in initiating early development is also supported by work in *Xenopus* embryos in which Laloo, the *Xenopus* ortholog of mammalian Fyn, appears to link FGF signaling at the cell surface to nuclear events required for mesoderm induction (9,10). In contrast, other work has shown a requirement for SFKs in the maintenance of self-renewal. ES cells carrying a targeted activating mutation in one allele of the SFK gene encoding Hck require reduced LIF concentrations for self-renewal (11). A correlation was reported between LIF-induced activation of Hck and ES cell renewal (12). The SFK member c-Yes has also been implicated in the activation of self-renewal pathways, because knockdown of c-Yes with silencing RNAs (siRNAs) leads to mES cell differentiation (13). Thus, individual SFKs control distinct and potentially opposing pathways in ES cell renewal and differentiation.

We previously proposed a model in which individual Src family members regulate either renewal or differentiation signaling pathways in mES cells with kinases controlling renewal pathways epistatic to those regulating differentiation pathways (7). In this model, when mES cells are grown in the presence of LIF, both renewal and differentiation pathways are active; however, self-renewal is observed due to the epistatic effect. Conversely, removal of LIF inactivates the self-renewal pathway, resulting in the loss of pluripotency. Selective inhibition of renewal kinases mimics growth in the absence of LIF, leading to differentiation. In contrast, simultaneous inhibition of both pathways suppresses renewal and differentiation, resulting in the observed differentiation block.

A prediction of our model is that singular restoration of differentiation-related SFK activity should induce mES cell differentiation in the face of overall SFK blockade. Here, we tested this prediction through the use of SFK alleles with engineered resistance to a pyrrolo-pyrimidine SFK inhibitor (A-419259), previously established to cause a reversible differentiation block in mES cells (7). Remarkably, the presence of a c-Src mutant resistant to this inhibitor reversed the differentiation block associated with inhibitor treatment, resulting in the formation of cells with properties of primitive ectoderm. This effect was unique to c-Src, as similar inhibitor-resistant mutants of Hck, Lck, c-Yes, or Fyn did not rescue the differentiation block. These results support the model in which individual SFKs regulate mES cell fate in opposing ways. Furthermore, they suggest that SFKs controlling renewal are epistatic to those regulating differentiation in mES cells, and that the formation of primitive ectoderm is dependent on the activity of c-Src.

RESULTS

Inhibitor-Resistant variants of Src Family Kinases retain catalytic activity

We applied a chemical genetics approach to investigate individual SFK functions in mES self-renewal and differentiation. Structural studies of the Src kinase family have provided insight into the mechanisms underlying the inhibition of SFK activity by ATP-competitive inhibitors. With this structural information, we developed catalytically active, but inhibitor-resistant mutants, of each Src family member. By expressing each mutant individually in mES cells in the presence of the inhibitor, we analyzed the biological activity of an individual SFK in an environment in which all endogenous SFK activity was suppressed.

The kinase domain structures of Hck, Src, Fyn, and Lck have been solved and are representative of the conserved bilobed structures of other kinase domains (14–17) (Fig. 1A). In each case, the N-lobe contains a five stranded β -sheet with a single α helix, and the C-lobe is comprised of six α -helices and two short β -sheets. A hinge region joins the lobes, allowing articulation between the two. The catalytic site lies in the region of the hinge and the ATP-binding pocket is composed of residues from both the N- and C-lobes. SFK members contain a hydrophobic cavity of approximately 100Å³ with an opening towards the back of the ATP-binding pocket. Crystallization of Hck with the pyrazolo-pyrimidine inhibitor PP1 revealed that accessibility

to this hydrophobic cavity provided the specificity for this class of inhibitors (18). PP1 and the closely related inhibitor PP2 bind to the catalytic site in a manner closely resembling that of the nonhydrolyzable ATP analog AMP-ANP (15,18). The amino groups of PP1 and PP2 form a hydrogen bond with the side chain hydroxyl group of the so-called “gatekeeper residue” Thr³³⁸, as well as with the main chain carbonyl of Glu³³⁹ (Fig. 1B) [all numbering based on structure of human c-Src (PDB:1FMK)] (16). A third hydrogen bond forms between the main chain amide of Met³⁴¹ and the inhibitor nitrogen at position 3. The orientation of the inhibitors places the 3-substituted phenyl moieties of both PP1 and PP2 partially in the hydrophobic cavity near the gatekeeper residue (Fig. 1C). Thus, the accessibility of this hydrophobic cavity to these phenyl substituents appears to confer specificity to this class of inhibitors.

This mechanism of inhibition and specificity is supported by comparison of the Hck:PP1 crystal structure to a model of PP1 in ATP-binding site of protein kinase A (PKA), a kinase insensitive to PP1 (18). In all SFKs, the entrance to the hydrophobic cavity is flanked by Thr³³⁸ and Ala⁴⁰³ (Fig. 1B and 1C). The relatively small size of these two residues creates an opening that allows access to the cavity by the methylphenyl group of PP1. In PKA, the two corresponding residues are Met¹²⁰ and Thr¹⁸³. Together, the larger sizes of these two residues diminishes access to the cavity, creating steric clash with the methylphenyl group of PP1. Based on this model, we reasoned that modifications of the homologous residues in SFKs that reduce access to the hydrophobic cavity should result in resistance to PP1 and PP2.

To test this idea, we constructed mutants of Fyn in which either Thr³³⁸ was substituted with methionine or Ala⁴⁰³ was substituted with threonine. In addition, because PP2-resistant kinases (for example, EGFR) often have a serine residue at the position corresponding to Ala⁴⁰³ of Fyn, we also generated a Fyn A403S mutant. We tested whether these mutations affected the kinase activity of Fyn in a 293T transient transfection assay in which the SFKs were coexpressed with signal transducer and activator of transcription 3 (Stat3) as substrate. Phosphorylation of Stat3 on Tyr⁷⁰⁵, which is readily detected with phosphospecific antibodies, reports SFK activity (19). In this assay, each of the Fyn mutants retained kinase activity comparable to that of wild-type Fyn (Fig. 1D).

We also used this assay to test the Fyn mutants for resistance to PP2. Transfected 293T cells were treated with PP2 or the dimethylsulfoxide (DMSO) carrier solvent for 24 hours. Immunoblot analysis showed concentration-dependent inhibition of Stat3 phosphorylation by wild-type Fyn, with substantial inhibition observed at 20 μ M PP2 (Fig. 1E). In contrast, the Fyn T338M mutant was completely resistant to PP2 even at 20 μ M. Similar analysis of the A403T and A403S mutants failed to show any decrease in sensitivity to PP2. These results demonstrate that SFK resistance to PP2 can be generated without affecting kinase activity by replacement of the gatekeeper residue with a bulkier methionine residue.

Mutation of the gatekeeper residue in SFK members confers resistance to A-419259

Although the X-ray crystal structures of SFKs bound to PP1 and PP2 provided a basis for the design of an inhibitor-resistant mutant of Fyn, experimental use of these inhibitors in ES cells is complicated by potential cross reactivity with other tyrosine kinases, most notably c-Abl, c-Kit, and PDGFR (20). The pyrrolo-pyrimidine inhibitor A-419259 demonstrates much greater specificity and potency towards SFKs, minimizing potential complications arising from off-target effects (21,22). Furthermore, this compound, like PP2, induces a differentiation block in mES cells while maintaining pluripotency, providing an ideal compound for our chemical genetics approach (7). Because the A-419259 pyrrolo-pyrimidine pharmacophore is very similar to that of PP1 and PP2 (Fig. 2A), we predicted that the PP2-resistant Fyn T338M mutant should be cross-resistant to this inhibitor as well. Indeed, when wild-type Fyn and the T338M mutant were expressed in 293T cells and exposed to increasing concentrations of A-419259, phosphorylation of Stat3 by wild-type Fyn was inhibited by as little as 100 nM A-419259 with

near complete inhibition at 300 nM, whereas the FynT338M mutant showed no decrease in Stat3 phosphorylation even when treated with 1 μ M inhibitor (Fig. 2B). Thus, the T338M mutation that rendered Fyn resistant to PP2 also renders this kinase resistant to A-419259, indicating that this compound also requires access to the hydrophobic cavity for its efficacy.

We next generated analogous T338M mutations for the SFKs c-Src, c-Yes, Hck, and Lck. We chose these kinases because of their previously described roles in mES cell self-renewal (Hck and c-Yes) (11,13), known constitutive activity in mES cells (c-Src and Fyn) (7), and transcriptional down regulation during differentiation (Hck and Lck) (7). Because the ATP-binding pockets of SFKs are highly conserved, we predicted that substitution of the threonine gatekeeper residue in each of these kinases with methionine would also result in resistance to inhibition by A-419259. We tested each of these T338M mutants for A-419259 sensitivity using Stat3 as a substrate in the 293T cell assay in comparison to the corresponding wild-type kinase (Fig. 2C). As was observed for Fyn, each of the wild-type kinases was inhibited by A-419259, with partial inhibition of Stat3 phosphorylation at 300 nM and complete loss of kinase activity at 1 μ M. Introduction of methionine at the gatekeeper position resulted in substantial resistance to A-419259, with no decrease in Stat3 phosphorylation observed at the highest concentration of inhibitor tested (1 μ M). Taken together, these results demonstrate that T338M mutations in SFKs result in complete resistance to ATP-competitive inhibitors of both the pyrazolo-pyrimidine (PP2) and pyrrolo-pyrimidine (A-419259) classes. We therefore designated these SFK mutants as inhibitor-resistant (IR) mutants.

With the exception of c-Src, mES cells expressing the inhibitor-resistant mutants exhibit undifferentiated growth

We next tested the effect of the SFK-IR mutants in mES cells using a transient transfection approach. Expression vectors for the SFK-IR mutants were designed to minimize the potential for false-positive results occurring as a consequence of kinase overexpression. The strategy to maintain near endogenous levels of kinase activity was twofold. First, we utilized the mouse stem cell virus (MSCV) promoter, which drives constitutive but a relatively low expression of heterologous genes in ES cells (23). Second, we avoided the use of any kinase-activating mutations, such as replacement of the regulatory C-terminal tyrosine residue. In this way, endogenous signaling pathways would be required to activate the transgenic kinases.

Each of the SFK-IR mutants was transiently expressed in the D3 line of mES cells. The transfected cells were grown overnight before the addition of A-419259 to a final concentration of 1 μ M, which has been previously shown to block all endogenous SFK activity in mES cells (7). Control cells were transfected with enhanced yellow fluorescent protein (EYFP) and in the absence of inhibitor exhibited growth of undifferentiated colonies of cells (Fig. 3A). Addition of 1 μ M A-419259 to the control culture resulted in the appearance of small rounded colonies, similar to those described previously, which consisted primarily of undifferentiated cells even in the absence of LIF (7). Twenty-four hours of A-419259 treatment of cultures transfected with Fyn-IR, Lck-IR, Yes-IR, and Hck-IR resulted in ES cell colonies morphologically indistinguishable from the inhibitor-treated control cells both in morphology and size (Fig. 3A and 3B). Cells transfected with the Src-IR mutant, in contrast, demonstrated a different morphology from the other transfected cultures. Within 24 hours of inhibitor addition, the Src-IR-transfected cultures had fewer rounded colonies of cells (Fig. 3A). Rather, Src-IR cultures were primarily comprised of flat adherent cells that appeared to have undergone differentiation. These flattened colonies continued to grow and nearly spread over the entire culture dish after 48 hours in the presence of the inhibitor.

We analyzed the abundance and activity of the SFK-IR mutants by immunoblotting of lysates from each transfected cell population with a kinase-specific antibody and with an antibody specific for the phosphotyrosine residue in the activation loop of the active form of each kinase,

respectively. In each case, transfection resulted in an increase in the abundance of the kinase protein compared to the control transfection (Fig. 3C). The relative increases in the abundance of the kinases from the control cells varied due to differences in the abundance of the endogenous kinases. Constitutive SFK activity in control mES cells was diminished by treatment with 1 μ M A-419259 (Fig. 3C, right), consistent with our earlier work (7). However, immunoblotting with the SFK phosphospecific antibody revealed that each of the SFK-IR mutants expressed in the transfected cells retained SFK activity in the presence of the inhibitor (Fig. 3C). Interestingly, the total SFK activity observed in cells transfected with Src-IR was greater than that observed for the other transfected kinases, which may reflect the constitutive activation previously described for c-Src in mES cells (7).

Stable expression of Src-IR results in the formation of primitive ectoderm-like cells in the presence of A-419259

To further characterize the effect of Src-IR on the mES cells, we generated a mES population stably expressing Src-IR. Expression of *Src-IR* was controlled by the MSCV promoter and linked to the *neo* resistance marker with an internal ribosome entry site (IRES) sequence so that *Src-IR* expression could be enforced by growth in G418 (Fig. 4A). As a control, a second cell population was created in which wild-type Src was expressed using an identical vector.

The presence of the transcripts for wild-type and Src-IR in the stable cell lines was confirmed in the transduced ES cell populations by reverse transcriptase-polymerase chain reaction (RT-PCR). To allow comparison of the relative expression of endogenous *Src* transcripts versus the two transduced alleles, we designed primer sets specific for the vector-derived transcripts (A primers) or that would detect endogenous and vector-derived c-*Src* transcripts (B primers) (Fig. 4A). Control PCR reactions confirmed the lack of genomic DNA in the cDNA samples (Fig. 4B). Amplification using primers specific to the transduced *Src* alleles confirmed vector-driven expression in both the c-*Src*-- and *Src-IR*--expressing cell populations (Fig. 4C). Finally, semi-quantitative RT-PCR was used to compare total abundance of *Src* transcripts in the two transduced cell populations compared to that in the parental ES cells. Comparison of the intensity of the *Src* bands amplified using the B primers showed only a slight increase in the abundance of the *Src* transcripts in the transduced cell populations as expected from these MSCV-driven constructs (Fig. 4D).

We examined the morphology of the ES cells stably expressing Src-IR in the presence of A-419259. ES cells stably expressing wild-type Src, Src-IR, or EYFP were plated in ES cell medium with LIF. After 24 hours, either 1 μ M A-419259 or DMSO was added, and the cultures were monitored for four days. Within 24 hours of inhibitor addition, the EYFP and wild-type Src control cell populations formed small rounded colonies of uniform size that did not expand during the remaining four days of inhibitor treatment (Fig. 4E). These results agreed with the transient expression experiments and are consistent with our previous observations of the effect of this SFK inhibitor on mES cells (7). In contrast, the ES cells expressing Src-IR showed a dramatic change in morphology within 24 hours of A-419259 addition: the culture was comprised primarily of flattened, single cells growing in a monolayer with very few rounded colonies. The cells in the inhibitor-treated Src-IR culture appeared to be homogenous and possibly of a single cell type.

We tested whether the morphological changes observed in Src-IR-expressing cells following inhibitor treatment were accompanied by changes in cellular pluripotency by performing quantitative real-time RT-PCR to assay four well characterized markers of pluripotency, *Oct4* (*Pou5F1*), *Nanog*, *Rex1* (*Zfp42*), and *Gbx2* (24–28). We also assayed *Klf4*, *Tcl1*, and *Tbx3*, three genes associated with pluripotency that are down regulated during embryo implantation (29,30). In addition, expression of the growth factor gene *Fgf5*, a marker of primitive ectoderm and one of the first markers of differentiation to appear as mES cells

differentiate, and the gene *T*, an early marker of mesoderm formation, were quantified to assess whether A-419259 treatment induced differentiation (31,32). Relative transcriptional changes for each of these markers were monitored in the parental D3 mES cell line, cells expressing wild-type c-Src, and cells expressing the Src-IR mutant following four days of treatment with 1 μ M A-419259.

With the exception of *Gbx2*, A-419259 treatment had little effect (less than a 3-fold change) on the expression of any of the tested genes in either the parent cell line or the c-Src-expressing cell line (Fig. 5A). Inhibitor treatment of these lines did result in at least a five-fold decrease in the relative abundance of the *Gbx2* transcript. The abundance of the *Nanog* transcripts were two- to three-fold higher than that of the untreated control cells, consistent with our previous results demonstrating that complete inhibition of SFK activity promotes self-renewal of mES cells (7). In contrast, cells expressing Src-IR had significant changes in the expression of multiple genes following inhibitor treatment. Like the control cells, *Gbx2* expression decreased to approximately 10% of the untreated cells. In contrast to the control lines, however, *Nanog* expression remained unchanged whereas *Rex1* expression decreased nearly three-fold relative to untreated cells. Inhibitor treatment of the Src-IR cells increased *Fgf5* expression by 5-fold, consistent with the onset of differentiation. Less than three-fold changes in expression were noted for the pluripotency-related genes *Klf4*, *Tcl1*, and *Tbx3* or the mesodermal gene *T*.

The down regulation of *Rex1* and *Gbx2* expression combined with the increase in *Fgf5* expression raised the possibility that these cells represent primitive ectoderm. Primitive ectoderm, the pluripotent tissue that gives rise to the three primary germ layers, develops from the inner cell mass following the formation of the proamniotic cavity (33). Formation of primitive ectoderm from mES cells has been recapitulated in vitro, resulting in early primitive ectoderm-like (EPL) cells with properties of true primitive ectoderm. Analysis of EPL cells has allowed the development of a genetic and biochemical profile that defines differentiation of EPL cells from mES cells (34). The expression of *Rex1* and *Gbx2* are both decreased during this transition whereas expression of the pluripotency marker *Oct4* remains unchanged between the two cell types and *Fgf5* expression increases (24,28,31,34).

Like primitive ectoderm, stem cells derived from the postimplantation epiblast retain pluripotency and have decreased expression of *Gbx2* and *Rex1* (35,36). To exclude the possibility that inhibitor-treated Src-IR cells are comprised of epiblast stem cell-like cells, we compared the changes in expression of *Klf4*, *Tbx3*, and *Fgf5* to published results comparing the expression of these genes in murine epiblast stem cells to mES cells. Epiblast stem cells express less *Tbx3* and *Klf4* relative to embryonic stem cells and have only a modest increase in *Fgf5* expression. This contrasts with our results that show no decrease in either *Tbx3* or *Klf4* and a significant increase in *Fgf5* expression in inhibitor-treated Src-IR cells (Fig. 5A). Thus, our results suggest that inhibitor-treated Src-IR cells exhibit properties of primitive ectoderm, not epiblast stem cell-like cells.

Alkaline phosphatase (AP) activity is a biochemical marker of pluripotency. Present in mES cells and in primitive ectoderm, AP activity rapidly declines during primitive ectoderm differentiation (34,37). To determine if the inhibitor-treated Src-IR cells were similar to EPL cells in this respect, total AP activity was quantified in the EYFP control and Src-IR cell populations after four days of treatment with either DMSO or 1 μ M A-419259. Treatment of the EYFP control cells with A-419259 resulted in nearly a 2-fold increase in AP activity relative to the DMSO-treated cells (Fig. 5B). The lack of an observed decrease in AP activity supports the hypothesis that Src-IR expression in the inhibitor treated cells does not drive differentiation beyond primitive ectoderm. The observed increase in AP activity is consistent with our previous observation showing that A-419259 inhibits mES cell differentiation and is likely the

result of fewer differentiated cells in the analyzed culture or may indicate regulation of AP activity by an SFK member other than c-Src

Src-IR cells recover the marker expression profile of mES cells after A-419259 washout

We next tested whether the genetic changes observed in the inhibitor-treated Src-IR cells represented irreversible differentiation or if the cells could revert to mES cells following removal of A-419259. Src-IR mES cells were grown with LIF in the presence or absence of A-419259 for four days, split, and then plated in ES cell medium without inhibitor. Consistent with previous results, DMSO-treated Src-IR cells retained the round colony morphology typical of mES cells whereas inhibitor-treated Src-IR cells assumed the flattened morphology described above (Fig. 6A). Within 48 hours of growth without inhibitor, the Src-IR cells reverted to the morphology of the DMSO-treated culture, re-establishing themselves as small rounded colonies typical of undifferentiated mES cells (Fig. 6A).

The rescued cultures were assayed for the expression of *Gbx2*, *Nanog*, *Oct4*, *Rex1*, and *Fgf5* using real-time quantitative RT-PCR. Expression of these genes in the two cultures was compared to expression in self-renewing Src-IR cells. Within two days of growth without inhibitor, the abundance of the transcripts for these markers returned to that of mES cells (Fig. 6B). The difference in the expression of each marker was less than two-fold relative to the control cells. Notably, transcripts for *Rex1* and *Gbx2*, the two genes down regulated during inhibitor treatment that are closely linked to EPL differentiation, were restored to amounts close to those of control mES cells. The expression of *Fgf5*, a gene up regulated during inhibitor treatment (Figure 5A), also returned to an expression similar to the control mES cells. These results establish that mES cells expressing Src-IR can reversibly transition between the pluripotent and primitive ectoderm states in response to inhibitor treatment.

Inhibitor treatment directs Src-IR cells towards a mesodermal fate in developing embryoid bodies

Embryoid bodies are highly organized structures that form when ES cells are cultured in suspension without LIF (38). Embryoid body formation recapitulates early development from the blastocyst through the egg cylinder stage (39,40). Thus during differentiation these structures rapidly lose pluripotent stem cells while gaining cells first with characteristics of primitive ectoderm and primitive endoderm and then of definitive endoderm, ectoderm, and mesoderm. We tested whether Src-IR cells grown in the presence of A-419259 retained full developmental potential to form embryoid bodies by comparing the expression of differentiation and pluripotency genes in embryoid bodies generated from inhibitor-treated wild-type and Src-IR cells. Wild-type or Src-IR cells were grown in the presence of either 1 μ M A-419259 or DMSO for four days. The cells were then harvested, washed free of inhibitor, and used to seed embryoid body cultures in the absence of inhibitor. Embryoid bodies were harvested at four days and markers of pluripotency and differentiation analyzed with embryonic stem cell quantitative real time PCR arrays. Changes in gene expression were calculated relative to expression in the corresponding cycling mES cell cultures (either wild-type or Src-IR mES cells), prior to four day treatment with inhibitor or DMSO.

Comparison of the expression of pluripotency markers showed little difference between embryoid bodies that developed from DMSO or inhibitor-treated wild-type or Src-IR cells (Fig. 7A). All EB populations showed substantial decreases in *Gbx2* and *Rex1* expression levels and nearly equivalent albeit lesser decreases in *Nanog*, *Oct4*, and *Sox2*. Thus, inhibitor treatment of either wild type or Src-IR cells had no discernable effect on the loss of mES cell pluripotency markers during EB development.

In contrast, the expression of markers associated with the development of primitive endoderm was affected by SFK inhibitor treatment regardless of the expression of Src-IR. Substantial increases in *AFP* expression and a slight increase *Gata4* expression observed in embryoid bodies derived from untreated cells were both diminished in embryoid bodies derived from inhibitor-treated cells (Fig. 7A). Similar changes were observed in the expression of *Sox17* in embryoid bodies derived from A-419259-treated Src-IR ES cells. Together, these results suggest the possibility that another SFK may regulate the expression of genes linked to the formation of primitive endoderm.

Transcriptional changes in genes associated with the appearance of differentiated tissue were also assayed in the developing embryoid bodies (Fig. 7B). Increases in the primitive ectoderm marker *Fgf5* were observed for all embryoid bodies. Expression of *Sox1*, an early marker of neural development was observed only in embryoid bodies derived from untreated wild-type cells. This finding suggests that c-Src activity may negatively influence the development of neural ectoderm. Note that expression of Src-IR suppresses *Sox1* expression independent of inhibitor treatment, consistent with this idea. Along these lines, expression of another neural marker (*Pax6*) was substantially decreased in Src-IR-derived embryoid bodies in the presence of inhibitor.

In contrast with the expression of markers of neural ectoderm, striking increases in the expression of markers of early mesoderm -- *Goosecoid* (*Gsc*) (7-fold), *FoxA2* (25-fold), and *T* (28-fold)-- were observed exclusively in embryoid bodies derived from inhibitor-treated Src-IR cells. Lesser increases were observed for markers representing mesodermal tissues, such as *Flt1* and *Cdh5* for endothelial cells, and *MyoD* for muscle, derived later in development. These results suggest that embryoid bodies generated from ES cells in which c-Src is the only active Src family member are predisposed towards the formation of mesoderm, a property also associated with EPL cells (41).

Discussion

We report here the use of a chemical genetic technique to define the specific role of c-Src activity in mES cell differentiation. This approach utilizes SFKs with engineered resistance to the broad spectrum SFK inhibitor A-419259. The SFK-IR mutants were expressed in mES cells and treated with a concentration of A-419259 previously shown to completely inhibit endogenous SFK activity, including the activities of Hck, Lyn, Fyn, c-Yes, Lck, Fgr, and c-Src (7). In this way, the activity of each individual SFK could be studied in mES cells in the absence of potentially redundant or epistatic functions of other Src family members. Using this approach, we made the unexpected discovery that sole expression of an inhibitor-resistant mutant of c-Src resulted in dramatic changes in cell morphology and differentiation marker expression following inhibitor treatment. None of these changes were observed in mES cells ectopically expressing wild-type c-Src from the same expression vector, indicating that the effects are attributable to sustained c-Src activity in the absence of other SFK activity. In contrast, expression of the IR mutants of four other Src family members had no observable effect on mES cell morphology or colony formation following inhibitor treatment, suggesting that c-Src activity is a key determinant of the transition from the pluripotent state to that of early ectoderm.

Inhibitor-treated Src-IR cells exhibited many similarities to EPL cells derived from ES cells by other techniques (34,42), as well as embryo-derived primitive ectoderm (43). Like our inhibitor-treated Src-IR cells, EPL cells have decreased expression of *Rex1* and *Gbx2* with little change in AP activity or *Oct4* expression relative to mES cells, can revert to a phenotype resembling mES cells, and show a predisposition towards the formation of mesoderm in developing embryoid bodies (34,41). One notable difference is that although embryoid bodies

generated from inhibitor-treated Src-IR cells failed to express markers of visceral endoderm, EPL cells retain this ability (41).

The role of SFKs in the regulation of mES cell pluripotency has been complicated by seemingly contradictory results. At least seven Src-family members are present in mES cells, making the study of individual kinases by inhibitor studies difficult (7). RNAi knockdown of one of these SFKs, c-Yes, results in a decrease in pluripotency and an increase in the expression of markers of differentiation (13), which suggests that c-Yes functions as a “renewal SFK” in mES cells. Alternately, complete blockade of SFK activity in mES cells with small molecule inhibitors, including A-419259, blocks mES cell differentiation (7). We have previously proposed a model that accounts for these seemingly contradictory results. In this model, mES cells have both renewal- and differentiation-linked SFKs. In pluripotent ES cells, renewal-related SFKs (for example, c-Yes) are active and epistatic to active differentiation-related SFKs (for example, c-Src). Under conditions that promote differentiation, such as LIF withdrawal, the renewal-related kinases are inactivated and the differentiation-linked kinases initiate the differentiation program. This simple model makes a very specific prediction: The presence of a differentiation-related SFK in the absence of renewal-related SFK activity should result in mES cell differentiation. The development of IR forms of SFKs allowed us to test this prediction directly. Our finding that singular restoration of c-Src activity, by expressing Src-IR in the presence of A-419259 treatment, resulted in differentiation of mES cells provides evidence that c-Src controls the differentiation commitment of mES cells.

There are at least two possible mechanisms by which Src-IR results in the differentiation of mES cells following treatment with A-419259: (i) Src-IR may activate primitive ectoderm differentiation pathways in mES cells, or (ii) Src-IR may promote the survival of primitive ectoderm cells once they form. Activation of cellular differentiation pathways by c-Src has been observed in several other cell lineages. For example, nerve growth factor-induced differentiation of the rat adrenal pheochromocytoma cell line PC12 and differentiation of the squamous epithelial cell line A431 both appear to be mediated by c-Src (44,45). Although the precise mechanism by which c-Src regulates differentiation in these cells is not clear, distinct differentiation pathways are likely to exist as Ras is involved in Src-mediated PC12 cell differentiation but not in that of A431 cells. Likewise, inhibition of apoptosis by Src is a well-described phenomenon, with v-Src expression resulting in the inhibition of apoptosis induced by various stimuli, including cytokine withdrawal, irradiation, and anoikis (46). However, identification of the signaling pathways involved has proved elusive and the use of an active, oncogenic form of Src in many of these experiments may not recapitulate normal c-Src function. Understanding the mechanisms by which Src-IR regulates differentiation in mES cells has the potential to provide insight into the developmental function of c-Src.

The development of the engineered inhibitor-resistant SFKs described here provides an approach to study the activity of a single Src family member in an environment devoid of other SFK activity. This chemical genetic approach has several advantages over RNAi-mediated knockdown or other genetic approaches. The selection step required for efficient knockdown of a single SFK may allow for compensatory changes in the expression or activity of other kinases, thus masking subtle phenotypic changes such as those observed here. In contrast, the SFK-IR approach allows for silent introduction of the modified kinase into the cells, followed by treatment with the global inhibitor to block all endogenous SFK activity. In this way, the cell does not have the opportunity for functional compensation. In addition, we are able to manipulate the activity of individual kinases by this approach, yet the abundance of the proteins themselves is unaltered (unlike RNAi knockdown). Thus, the results derived from our study are directly applicable to the development of new SFK inhibitors that could be used to guide specific fates of ES cells provided they possess the appropriate kinase target inhibitory profiles. Based on the results presented here, a Src-sparing inhibitor that retains activity against other

family members may push ES cells towards primitive ectoderm and possibly mesoderm. Development of such compounds may ultimately be an asset for stem-cell based therapies, as they could enable non-genetic approaches to enrich progenitor cell populations (e.g. mesodermal lineages) for differentiation to cell types ultimately used in transplantation (e.g. cardiomyocytes).

Materials and Methods

Inhibitors

PP2 was purchased from Calbiochem. A-419259 was provided by Abbott BioResearch, Worcester, MA (21).

Embryonic stem cell culture

The D3 line of embryonic stem cells was obtained from the American Type Culture Collection (ATCC) (47). Cells were 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 (48).

Immunoblot analysis

Immunoblotting was performed as described (7). Antibodies used were Src phosphospecific (pY418), Biosource International KHO0171; c-Src, Santa Cruz Biotechnology (SCBT) sc-18; Fyn, SCBT sc-16; Lck, Signal Transduction Labs 15269; Yes, (SCBT) sc-8403; Hck, SCBT sc-1428; Stat3, SCBT sc-483; and Stat3 (pY705), Millipore 05-485.

Analysis of SFK inhibitor resistance in 293T cells

The expression constructs for murine cDNA clones of c-Src, Fyn, Lck, and Hck were described previously (7). The cDNA clone for murine c-Yes was obtained from Open Biosystems (MMM1013-65192). All clones are under regulation of the CMV promoter in either pCDNA3.1 (Fyn, Lck, Hck) or pSPORT6 (c-Src, Yes). Site-directed mutagenesis of SFK members was accomplished using the Stratagene QuickChange XL kit according to the protocol of the manufacturer. In each case, mutagenesis of the respective allele was confirmed by sequence analysis. Vectors encoding wild-type or mutant SFKs were transiently transfected in combination with human Stat3 into human 293T cells as described elsewhere (49). After 18 h, inhibitor was added and the cells were allowed to grow for another 24 h before immunoblot analysis.

Transient expression of SFK inhibitor-resistant (IR) mutants in mES cells

Plasmid constructs for the transient expression of SFK-IR mutants in mES cells utilized the vector pMSCV-Neo (Clontech). ES cells were transfected using a modification of a published protocol and Lipofectamine 2000 (50). The ES cells to be transfected were grown for a minimum of 18 h in ES cell medium without antibiotic. Immediately prior to transfection, 250 μ l OptiMem was placed in two sterile 1.5-ml tubes. Four micrograms of the DNA to be transfected was added to one tube and 10 μ l Lipofectamine 2000 was added to the second tube. After 5 min at room temperature, the contents of the two tubes were combined and incubated for 20 min further. A total of 1.5×10^6 ES cells were suspended in the diluted transfection mixture for 10 min, plated in 6 ml of ES cell medium without antibiotic, and grown overnight. Pilot transfections with a pMSCV:EYFP construct showed this protocol to yield a transfection efficiency of approximately 65%.

Stable expression of c-Src and Src-IR in mES cells

To create the stable expression constructs, the Neo^R coding region was amplified from pMSCV-Neo (Clontech) and subcloned into the pIRES expression vector (Clontech) downstream of the IRES sequence. This IRES- Neo^R construct, including the SV40 polyA+ signal sequence, was then used to replace the PGK-Neo selection cassette in pMSCV-Neo. EYFP, c-Src or Src-IR genes were then cloned into this vector upstream of the IRES sequence. The resulting retroviral expression constructs thus contained these three cDNAs linked to Neo^R by an IRES sequence under the regulation of the MSCV promoter. Production of recombinant retroviruses and infection of D3 ES cells were as described (23). Cells were selected and maintained in 200 µg/ml G418.

RT-PCR

Total RNA was isolated using the RNeasy kit in conjunction with QiaShredder columns (Qiagen). For semi-quantitative analysis, cDNA was synthesized from 2 µg of total RNA using MMLV reverse transcriptase and the Ambion Retroscript kit. One-fifteenth of each RT reaction was then used in 50 µl PCR reactions. Aliquots (5 µl) of each reaction were run on 2% agarose gels and stained with ethidium bromide.

For quantitative PCR analysis, cDNA was generated from 1 µg of total RNA using the RT² First Strand Kit from SuperArray Bioscience Corporation. PCR reactions were performed using the RT2 SYBR/Rox Master Mix from SuperArray using 1 µl of a 1:100 dilution of the cDNA reaction. Measurements were made on an ABI 7900HT instrument. Transcript abundances were calculated using RT reactions from self-renewing ES cells as the calibrator sample and *GAPDH* as the internal control (51,52). Results for *Oct4*, *Nanog*, *Gbx2*, *Fgf5*, *Klf4*, *Tcl1*, *Tbx3*, *T*, and *Rex1* were calculated using the Relative Expression Software Tool and statistical significance was determined using the Pair-Wise Fixed Reallocation Randomization Test (53). Primers used are the commercially available Quantitec Primer Assays from Qiagen. Primer efficiency was calculated using serial dilutions of an equal combination of cDNA generated from mES cells and six day embryoid bodies. Primer efficiencies were calculated as GAPDH, 100%; Nanog, 103%; Rex1, 99%; Oct4, 98%; Gbx2, 103%; Fgf5, 103%; Klf4, 103%; Tcl1, 89%; Tbx3, 96%; T, 98%.

PCR array analysis was performed using the murine embryonic stem cell RT2 Profiler Arrays from SABiosciences. cDNA was generated as described above. Array results were analyzed using the online software provided by the manufacturer (<http://www.sabiosciences.com/pcr/arrayanalysis.php>).

Alkaline phosphatase activity

Alkaline phosphatase activity was assayed using the ALP 10 alkaline phosphatase activity reagent from Sigma Diagnostics (#245-10). Following trypsinization and a single wash with PBS, 2×10^5 cells were lysed in 100 µl of a 0.1% Triton solution. Twenty µl of the cell lysate was added to 1 ml of reagent for the assay. Absorbance at 405 nm was recorded every 2 min for a total of 10 min. ALP activity was then quantified as $\Delta\text{ABS}_{405}/\text{min}$.

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Fig. 1. Design of SFK inhibitor-resistant (IR) mutants

(A) Crystal structure of the kinase domain of Hck bound to the pyrazolo-pyrimidine inhibitor, PP1 (PDB: 1QCF). The N-lobe of the kinase domain is shaded blue; the C-lobe is colored in green. Residues involved in inhibitor binding are colored red; this region is expanded in part C. A space-filling model of PP1 is shown and maps the inhibitor binding site. (B) Structures of the SFK inhibitors PP1 and PP2. Dashed lines represent hydrogen bonds between the ligands and kinase domain active site residues, which are conserved in all Src family members. Numbering of amino acid residues is based on that of the human c-Src crystal structure (PDB: 1FMK). (C) Surface plot of the active site of Hck bound to inhibitor PP1. Surface areas of the gatekeeper residue Thr³³⁸ as well as Ala⁴⁰³ are represented in red and yellow, respectively.

The methylphenyl moiety of PP1 projects into the hydrophobic pocket. **(D)** Kinase activity of Fyn Thr³³⁸ and Ala⁴⁰³ mutants. Wild-type Fyn and the Fyn mutants indicated were co-expressed with Stat3 in 293T cells. Cell lysates were analyzed for expression of Fyn and Stat3 and for phosphorylation of Stat3 at Tyr⁷⁰⁵ by immunoblotting. **(E)** The FynT338M mutant is resistant to PP2. 293T cells were co-transfected with the indicated Fyn mutants and Stat3, and PP2 was added to the cultures at the indicated concentrations 18 h later. Cell lysates were harvested 24 hours after inhibitor addition and analyzed by immunoblotting for kinase and Stat3 abundance, as well as Stat3 tyrosine phosphorylation as a measure of kinase activity.

Fig. 2. SFK T338M mutants are resistant to the broad-spectrum SFK inhibitor, A-419259

(A) Structure of the pyrrolo-pyrimidine SFK inhibitor, A-419259. (B) FynT338M is resistant to A-419259. 293T cells were co-transfected with Stat3 and either wild-type Fyn or FynT338M. After 18 hours, A-419259 was added to the indicated concentrations. The kinase activity of Fyn was assayed by immunoblotting for phosphorylation of Stat3 on Tyr⁷⁰⁵ in cell lysates 24 h later (pStat3). Control blots show the abundance of Stat3 and Fyn in each lysate. (C) T338M mutants of c-Src, Lck, c-Yes, and Hck are resistant to A-419259. 293T cells were co-transfected with Stat3 and wild-type or T338M mutants of the indicated kinases. Kinase activity was assayed as Stat3 phosphorylation as in B.

Fig. 3. A-419259 treatment of mES cells expressing SFK-IR mutants

(A) The D3 line of mES cells was grown in the presence of LIF and transiently transfected with either EYFP or the indicated SFK-IR mutants. A-419259 was added to a final concentration of 1 μ M 18 h later. Control EYFP-transfected cultures were treated either with inhibitor or with 0.1% DMSO (No A419). The transfected cultures were photographed 24 hours after the addition of DMSO or inhibitor, with the exception of the Src-IR culture, which was imaged after 24 h and 48 h (as indicated). (B) Size of transfected colonies following 24 hours of treatment with A-419259. Colony area was determined using ImageJ (<http://rsb.info.nih.gov/ij/>). At least 75 colonies were measured from two separate experiments for each of the transfected kinases. To allow comparison, individual experiments were normalized to a control (EYFP without A-419259) colony size of 1000 (arbitrary units). Colony data are not presented for c-Src-IR cells because these cells flatten out and grow in monolayer. (C) Analysis of kinase abundance and activity in mES cells transiently expressing either EYFP (Con) or the SFK-IR mutants. Protein lysates were prepared from each of the cultures shown in part A, and blotted with antibodies to Fyn, Lck, Src, Yes, and Hck as shown (left blots). SFK activity was assayed by immunoblot analysis using a phosphorylation-specific antibody that recognizes the phosphorylated activation loop of each SFK (pSFK; arrow). Lysates were also probed for actin as a loading control (right blots).

Fig. 4. Stable expression of a Src-IR:IRES:Neo construct in mES cells

(A) Map of the retroviral expression construct, showing the relative orientation of the MSCV promoter, Src cDNA clone (kinase), the IRES, and the Neo selection marker. Two sets of primers were utilized for analysis of Src expression. The “A primers” amplify 2.0 kb transcripts specifically derived from the expression constructs. The “B primers” amplify a 490 base pair (bp) fragment of Src transcripts derived from both the expression construct and the endogenous *c-Src* locus. (B) Equivalent amplification of HPRT from control and the two Src-expressing ES cell lines. RNA was isolated from each of the three cell lines, and first-stand reactions were run in the presence or absence of reverse transcriptase. HPRT primers were then used to amplify these control transcripts from each sample (HPRT PCR product is 349 bp). (C) Specific expression of Src from the retroviral constructs in mES cells. The “A” primers were used in RT-PCR reactions with RNA isolated from control cells, as well as the mES cell lines expressing wild-type Src (Src-WT) or the Src-IR mutant. The PCR reaction was allowed to proceed for 30 cycles. (D) RT-PCR comparison of overall Src transcript abundance. The “B” primers were used to amplify a 490 bp region of Src transcripts from each of the three cell lines. PCR reactions were analyzed after 27, 31, and 35 cycles to permit semi-quantitative analysis. HPRT was amplified as a positive control. (E) Morphology of mES cells stably expressing wild-type Src or Src-IR in the presence or absence of A-419259. mES cells stably expressing wild-type Src, the Src-IR mutant, or EYFP as a negative control were grown in the presence of either 0.1% DMSO or 1 μM A-419259 for 24 hours and photographed.

Fig. 5. Effect of inhibitor treatment on expression of pluripotency and differentiation markers in Src-IR cells

(A) Cells grown in the presence of LIF were treated with 1 μ M inhibitor for four days. RNA was then isolated and analyzed for the expression of *Gbx2*, *Nanog*, *Oct4*, *Rex1*, *Klf4*, *Tbx3*, *Tcl1*, *T*, and *Fgf5* using quantitative real-time RT-PCR. Expression of each gene is normalized to GAPDH expression and results are presented relative to the expression of each gene in the corresponding mES cell line prior to inhibitor treatment. Columns depict the mean change in expression $\pm$ 95% confidence intervals. *, $p < 0.05$; Pairwise Fixed Reallocation Randomization Test. At least three independent experiments were performed. (B) Control and Src-IR cells were treated with either 0.1% DMSO or 1 μ M A-419259 for four days. Cells were then harvested and analyzed for total alkaline phosphatase (AP) activity. Results are reported relative to the AP activity of the DMSO treated cells ($n=3$).

A **B**

Fig. 6. Rescue of Src-IR-induced mES cell differentiation by washout of A-419259

(A) Src-IR cells grown in the presence of LIF were treated with either 0.1% DMSO or 1 μ M A-419259 for four days. Cells were then washed with PBS, trypsinized, and plated in fresh medium lacking inhibitor. Photographs were taken 48 h after the addition of inhibitor or DMSO (Treatment) and re-photographed 48 h after removal of inhibitor or DMSO (Washout). (B) Src-IR cells were treated with A-419259 and rescued as described in A. After 48 hours, expression of *Gbx2*, *Nanog*, *Oct4*, *Rex1*, and *Fgf5* was analyzed by real time RT-PCR. Results shown are normalized to expression levels in untreated Src-IR cells. Values shown represent the mean ratios $\pm$ S.D. (n=3).

Fig. 7. Inhibitor-treated Src-IR cells are predisposed to generate mesoderm during in vitro embryoid body development

(A) Embryoid bodies (EB) were generated from wild-type D3 mES cells (D3) or Src-IR mES cells grown in the presence of either DMSO or 1 μ M A-419259 (+ A4) for four days. The expression of markers for self-renewal and primitive endoderm formation in the developing EBs was determined by quantitative real time RT-PCR. Expression analysis of all genes was done using PCR arrays as described under Materials and Methods using GAPDH as the internal control. Results are shown as fold-change relative to either self-renewing control D3 mES cells or Src-IR mES cells. $n \geq 2$; (B) Analysis of markers of ectoderm and mesoderm formation in four-day EBs was performed as described in part A.