The Src Family of Tyrosine Kinases Is Important for Embryonic Stem Cell Self-renewal*

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Embryonic stem (ES) cells are derived directly from the inner cell mass of a blastocyst. They can be expanded in culture and retain attributes of pluripotent cells of early embryos when reintroduced into the blastocyst. The capacity for multilineage differentiation can be reproduced in vitro, wherein ES cells can be induced to differentiate into a variety of cell types (reviewed in Refs. 1 and 2). There is currently considerable interest in the prospect of exploring this potential to provide a renewable source of material for cell therapy in regenerative medicine. One of the most important issues in stem cell biology is the mechanism that regulates self-renewal, a crucial aspect of stem cell function (reviewed in Ref. 3). The propagation of pluripotent mouse embryonic stem cells depends upon the cytokine leukemia inhibitory factor (LIF), which activates gp130 via dimerization with the LIF-specific receptor subunit LIFRβ (4, 5). Gp130 is known to activate the Janus-associated tyrosine kinases (JAK)/signal transducer and activator of transcription (STAT)3 pathways. Several lines of evidence suggest that STAT3 activation is necessary and sufficient for maintaining ES cells in an undifferentiated state (6, 7), whereas the MAPK pathway antagonizes ES cell self-renewal partly because of a negative feedback on JAK activity (8, 9). The overall balance of conflicting activation of STAT3, extracellular signal-regulated kinase (ERK)1 and 2, and MAPKs might determine the efficiency of ES self-renewal. The signals that maintain ES cells in an undifferentiated state are, however, not fully understood, and a recent report shows that bone morphogenetic proteins may act in combination with LIF to sustain self-renewal (10).

There are a few genes whose functions are known to be essential for ES cell pluripotency, including the POU family transcription factor Oct3/4 (octamer binding protein 3 or 4 encoded by Pou5f1; reviewed in Ref. 3), which is down-regulated in response to differentiation. However, exogenous expression of Oct3/4 does not prevent ES cell differentiation induced by LIF withdrawal and a less than 2-fold increase in Oct3/4 expression causes differentiation, all of which suggests that there are other genes controlling ES cell maintenance and pluripotency (11). Recently, the homeo domain protein Nanog was shown to be capable of maintaining ES cell self-renewal independently of the LIF/STAT3 pathway (12, 13).

In contrast to mouse ES (mES) cells, human ES (hES) cells do not seem to require LIF for maintenance in vitro. It has been suggested that the discrepancy between mouse and human ES cells may be due to the surface markers/receptors they express, whereas the downstream signaling pathways, such as the JAK/STAT3 pathway, may be conserved (14).

Our attention was drawn to the Src family after examining transcriptional profiles for mouse embryonic, hematopoietic, and neuronal stem cells, which showed that mRNA for the Src-family member, cYes, is enriched in all of these stem cell populations compared with their differentiated counterparts. Notably, the enrichment of cYes transcripts is most pronounced in ES cells (15, 16). The Src family of non-receptor protein tyrosine kinases regulates diverse processes such as cell division, motility, adhesion, differentiation, and survival (17–19). The Src family has also been implicated in the maintenance of ES cells by the fact that LIF activates the Src family member Hck (20), and expression of kinase active mutants of Src and Hck can maintain ES cells in an undifferentiated state when LIF concentrations are reduced but not absent (21, 22). What has not been determined heretofore is whether Src family members are necessary for ES cell maintenance of the undifferentiated state, nor have the downstream signaling pathways by which these kinases exert their effects been established.

To address the role of the Src family, and in particular

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‡ The abbreviations used are: ES, embryonic stem; LIF, leukemia inhibitory factor; JAK, Janus-associated tyrosine kinases; STAT3, signal transducer and activator of transcription 3; p42/p44 MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; mES, mouse ES; EBs, embryoid bodies; siRNA, small interfering RNAs; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; RFP, red fluorescent protein; RT, reverse-transcription; GCNF, germ-cell nuclear factor; FGF, fibroblast growth factor; MEF, mouse embry fibroblast; AP, alkaline phosphatase; RA, retinoic acid.
EXPERIMENTAL PROCEDURES

Materials—Antibodies were purchased from the following vendors: monoclonal anti-cYes, BD Transduction Laboratories; anti-phospho-Src (Tyr527) and anti-phospho-p44/p42 (Thr202/Tyr204) MAPK (ERK1/2) antibodies, Cell Signaling Technology; anti-pi3K (20C3) antibody (Cell Signaling Technology); and monoclonal anti-neuronal class III β-tubulin antibody, Covance Research Products; polyclonal anti-human α-1-fetoprotein (AFP) antibody, Dako; monoclonal anti-BrdUrd antibody (G34), Developmental Studies Hybridoma Bank; and monoclonal anti-phosphotyrosine antibody (4G10), Upstate Biotechnology. All-trans-retinoic acid was obtained from Sigma and SU6656 inhibitor was purchased from Calbiochem.

Cells—Experiments were performed using the H1 mouse ES cell line derived from the 129sv strain (a kind gift from Andrew McMahon, Harvard University) passage 14–40. All results were confirmed in the C57Bl6/6-derived ES cell line (a kind gift from Colin Stewart, NCI/ National Institutes of Health, Frederick, MD). Cells were cultured as described previously (25). The H9 human ES cell line, passage 19–40, was cultured as described previously (24). All results were confirmed in a new hES cell line (25). Before stimulation with LIF or serum, the mouse embryo fibroblast (MEF)-depleted cells were cultured overnight in serum- and LIF-free ES media. The SU6656 inhibitor (2.5 mM stock in DMSO) was added 30 min prior to LIF.

Cells were plated on gelatin or cultured in ES media containing 500 μM LIF and different concentrations of SU6656, and the number of cells, as a percentage of the untreated control, were counted on day 4.

Alkaline Phosphatase Staining—Cells were cultured for 6 days under different culture conditions and stained with the alkaline phosphatase substrate kit (Vector Laboratories). The proportion of undifferentiated cells was determined by scoring at least 300 randomly chosen ES cell colonies. Colonies consisting entirely of densely stained alkaline phosphatase-positive cells were scored as “undifferentiated,” whereas colonies consisting of a mixture of unstained and stained cells or entirely of unstained cells with flattened irregular morphology were scored as “differentiated.”

Cell Viability—The proportion of apoptotic cells was analyzed using the DeadEndTM fluorometric terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) system (Promega) of trypsinized cells and flow cytometry according to the manufacturer’s instructions. The proportion of necrotic cells was determined by staining live cells with 20 μg/ml Hoechst 33342 and 10 μg/ml propidium iodide for 5 min at 37 °C. Cells were then trypsinized, washed, and immediately subjected to analysis by flow cytometry.

Flow Cytometry and Immunohistochemistry for Oct3/4—Mouse ES cells, cultured for 8 days with different treatments, were trypsinized, washed with cold phosphate-buffered saline (PBS) + 1% fetal bovine serum (FBS), and fixed for 15 min in 4% paraformaldehyde. The cells were permeablized in 0.1% Nonidet P-40 + 5% donkey serum in PBS for 30 min followed by incubation with monoclonal Oct3/4 antibody (1:200) overnight at 4 °C and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse antibody (The Jackson Laboratory) for 1 h at room temperature. Cells were re-suspended in 0.5 ml PBS + 1% FBS, filtered by flow cytometry using a triple laser MoFlo (Cytometry) using the Summit software.

Immunohistochemistry—Mouse ES cells cultured under various conditions on gelatin-coated dishes were fixed for 15 min with 4% paraformaldehyde and permeabilized in 1% Triton X-100 and 2% Nonidet P-40 in PBS for 10 min. Nonpecific binding was blocked by incubation with 20 μg/ml donkey serum in PBS-T (0.2% Nonidet P-40 + 0.1% Triton X-100 in PBS) for 60 min. Cells were incubated with a monoclonal anti-TUJ1 antibody (1:500) and a polyclonal anti-AFP antibody (1:500) overnight followed by incubation with a rhodamine-conjugated donkey anti-mouse secondary antibody and an FITC-conjugated donkey anti-rabbit secondary antibody. Mouse ES cells cultured under various conditions on gelatin-coated dishes were incubated with 10 μl BrdUrd for 20 min and fixed with paraformaldehyde. The cells were permeabilized with 4 N HCl in 0.1% Triton X-100 for 10 min followed by several washes with PBS-T, and nonspecific binding was inhibited by incubation with 15% donkey serum in PBS-T. Cells were then incubated sequentially with a monoclonal anti-BrdUrd antibody (G34; 1:100 diluted in blocking buffer) and a FITC-conjugated donkey anti-mouse secondary antibody. At least 1000 cells were counted to assess the percent BrdUrd-positive cells.

Western Blot Analysis on Cell Lysates—Subconfluent cells were washed with cold PBS, briefly sonicated in SDS-sample buffer (containing β-mercaptoethanol and 2 μm phenylmethylsulfonyl fluoride), and centrifuged to remove debris. The membrane was probed with the indicated antibodies, and the immunoreactivity was subsequently detected by the enhanced chemiluminescence (ECL) immunoblot detection system (Amersham Biosciences).

In Vitro Kinase Assay—Mouse ES cells cultured to sub-confluence on 10-cm dishes or equal amounts of ES were lysed in 150 μl NaCl, 30 μl Tris, pH 7.5, 10 μl EDTA, 1 N Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μM orthovanadate, 2 μm phenylmethylsulfonyl fluoride, 100 units/ml aprotinin, 5 μm sodium fluoride, and 0.05 μM leupeptin, and the nuclei were removed by centrifugation. cYes was immunoprecipitated with 1 μg of each of anti-Yes antibody and protein A-Sepharose and subjected to an in vitro kinase reaction by incubation in kinase buffer (50 μl) for 30 min, 10 μg C/EBP, 5% glyceral, 1 mM dithiothreitol, and 7 μCi [γ32P]ATP for 15 min in room temperature. The reaction was stopped by the addition of 70 μl of SDS-sample buffer with β-mercaptoethanol. Proteins were separated on a 7.5% SDS-polyacrylamide gel and subjected to Western blotting for cYes and phospho-Src (Tyr527) or autoradiography.

Expressing Small Interfering RNAs (siRNA) in Mouse ES Cells—Expression of siRNA was achieved by transfection of pSilencer™ 1.0-U6 siRNA expression vector (Ambion) driving the expression of specific siRNA. Each construct was sequenced before transfection. The oligonucleotides designed for RNAi were as follows: 5′-GATTCGCCAAAGAGATACTCTTCTCTCCAGTTCTCCCTTTTTT-3′ and 5′-TTGTACCTGGAGAAAAAGAGTGTTCTCAAGACACTTTTCCCGTGACACCC-3′ (yes1); 5′-GATTCGCCAAAGAGATACTCTTCTCTCCAGTTCTCCCTTTTTT-3′ and 5′-TTGTACCTGGAGAAAAAGAGTGTTCTCAAGACACTTTTCCCGTGACACCC-3′ (yes2); 5′-CGATCTGGAAGCCCTTTTCAACAGAGAGAGGCACACTTCCCTTTTTTT-3′ and 5′-AATTAAAAAAAGTTTCAAGAGCAGGAAAGTGCTTGGACTGACCTACCTTTCCCGTGACACCC-3′ (Nanog); and 5′-GAAGAGCAGCAGCAGGAACTTTCTTCCTTCCTAAGAAGCTAGCAGGAAATTTTTTT-3′ and 5′-AATTAAAAAAAGTTTCAAGAGCAGGAAAGTGCTTGGACTGACCTACCTTTCCCGTGACACCC-3′ (enhanced green fluorescent protein (EGFP)). The siRNA constructs were co-transfected with a puro-GEFP vector containing the chicken α-actin (CBA) promoter driving the puro-GEFP transgene fused to GFP and linked to a puromycin-resistance gene by internal ribosomal entry site (pTP6; a kind gift from Andrew McMahon, Harvard University) at a ratio of 1:20 using LipofectAMINETM 2000 (Invitrogen). The pRed2-N1 vector (Clontech) was used to express red fluorescent protein (RFP). Transfected cells were cultured in selection ES media supplemented with 3 μg/ml puromycin (Sigma) for 14 days. Colonies were stained with alkaline phosphatase as described above, and the number of colonies as a percentage of control (EGFP siRNA transfected cells) were counted.

Semi-quantitive Reverse-transcription (RT)-PCR and Real-time PCR—Total RNA, extracted from cells using the RNAeasy mini kit (Qiagen), was reverse transcribed into cDNA in 30 μl reaction. cDNA was subjected to RT-PCR as follows: 1–4 μg of RNA were converted to cDNA by the SuperScript™ first-strand synthesis system (Invitrogen). Reactions not catalyzed by reverse transcriptase were run in parallel. 0.1–1 μl of the cDNA was added to each 25-μl PCR reaction using Taq polymerase (Promega) and the following primers: 5′-CACAAGAGGACACTCTCAGG-3′ and 5′-GAAGAGCAGCAGCAGGAACTTTCTTCCTTCCTAAGAAGCTAGCAGGAAATTTTTTT-3′ (Nanog); 5′-GAAGAGCAGCAGCAGGAACTTTCTTCCTTCCTAAGAAGCTAGCAGGAAATTTTTTT-3′ and 5′-CACAAGAGGACACTCTCAGG-3′ (cripto); 5′-GTGGTTTTGGGTTTGCTGGGTTTTT-3′ and 5′-TGTTCTCTCTGGAGTTCTTG-3′ (microphthalmia associated transcription factor (MITF)); 5′-GGCCGAGAGACACCTATTTG-3′ and 5′-GAAGAGCAGCAGCAGGAACTTTCTTCCTTCCTAAGAAGCTAGCAGGAAATTTTTTT-3′ (Ac5aR); and 5′-CACAAGAGGACACTCTCAGG-3′ (β-actin).
been treated with reverse transcriptase were run in parallel to verify that the obtained PCR products were amplified cDNA with no contamination of genomic DNA (results not shown). Densitometric scannings of the PCR bands were performed using the object image 2.08 f software.

Real-time quantitative PCR was performed on 0.1 μg cDNA using the QuantiTect SYBR green PCR kit (Qiagen) and DNA Engine Opticon (MJ Research) according to the manufacturers’ instructions.

RESULTS

Expression Levels and Kinase Activity of cYes in Mouse and Human ES Cells—To determine whether cYes protein expression or activity was regulated in ES cells under different conditions, Western blotting, and in vitro kinase assays were performed on undifferentiated mouse and human ES cells as well as on 10-day-old mouse and human EBs. As shown in Fig. 1A, cYes is highly active in both undifferentiated human and mouse ES cells, as assessed by autophosphorylation of cYes. cYes activity was significantly down-regulated when mouse ES cells differentiated and the decreased cYes activity could be detected as early as 4 days after LIF withdrawal. By contrast, the level of cYes protein was unchanged or only marginally reduced in mouse EBs cultured for up to 21 days in the absence of LIF (data not shown). To evaluate whether cYes is part of an LIF signal transduction pathway, as shown for Hck (20, 22), mES cells were serum-starved overnight and stimulated with different concentrations of LIF for various periods of time (C). Cells cultured in serum with 500 pm LIF were run in parallel as a control (B, 500 on). cYes autophosphorylation was assessed as above. D, the selective Src family inhibitor SU6656 inhibits LIF-induced activation of cYes in mES cells. mES cells were serum-starved overnight and stimulated with 32 nm LIF for 20 min with or without the presence of 0.5, 2, or 5 μM SU6656, which was added 30 min prior to LIF; cYes kinase activity was then assessed. E, mouse ES cells were serum-starved overnight and treated with serum (15% FBS) or 32 nm LIF for 20 min; cYes kinase activity was then assessed. Western blots were probed with anti-cYes and anti-phospho 416 Src antibody.

Serum-starved mES cells treated with 15% FBS for 20 min also exhibited increased cYes kinase activity compared with untreated cells. The degree of cYes-activation in response to serum was similar to that following LIF activation (Fig. 1E). SU6656, at concentrations above 0.5 μM, inhibited the LIF-dependent activation of cYes in mES cells (Fig. 1D). The SU6656 inhibitor potently and selectively inhibits cYes, cSrc, Fyn, and Lyn of the Src family. In contrast to other frequently used Src family inhibitors, SU6656 does not inhibit non-Src family tyrosine kinases such as PDGFRβ, FGFR1, IGF1R, Met, Csk, Kit Bcr-Abl, or Cdk2, nor does it inhibit the Src family members Lck or Frk/Rak (23, 26). It is unclear whether SU6656 inhibits Hck kinase activity.

In summary, cYes protein is easily detected in both mouse and human ES cells, and its kinase activity is activated by LIF and it is serum- and down-regulated when cells differentiate.

The JAK1/STAT3 and Ras/MAPK Pathways Are Not Disrupted by Src Family Suppression in Mouse ES Cells—To assess if the JAK/STAT3 or MAPK pathways are activated down-
stream of the Src family in response to LIF, we treated mES cells with 5 μM SU6656 and/or LIF and studied the phosphorylation of JAK1 and STAT3. Treating cells with the kinase inhibitor mediated no immediate changes in LIF-induced STAT3 or JAK1 tyrosine phosphorylation, as assessed by immunoprecipitation with anti-JAK1 or anti-STAT3 antibodies and blotting for tyrosine-phosphorylated proteins (Fig. 2A). The data was confirmed by immunoblotting cell lysates with anti-phospho Tyr705 STAT3 antibody (results not shown). Because it has been reported that high levels of SU6656 (20 μM) for a prolonged culture period (24 h) can reduce STAT3 activity in A549 lung cancer cells (27), mES cells were cultured for 24 h in 0, 2, 5, or 20 μM SU6656, and STAT3 tyrosine phosphorylation was assessed (Fig. 2B). No detectable effects upon STAT3 phosphorylation or protein levels were observed when cells were treated with 2 μM SU6656, a concentration shown to efficiently inhibit Src family kinases. A reduction in the level of STAT3 phosphorylation, proportional to a concomitant decrease in total STAT3 protein levels, was observed when cells were cultured in 5 and 20 μM SU6656, concentrations that are toxic in ES cells (Fig. 2B and results not shown). Given that previous reports have shown that such high concentrations of SU6656 may induce unspecific inhibition of non-Src family kinases, we conclude that inhibition of the Src family does not affect the STAT3 pathway in ES cells. In addition, the Ras/MAPK pathway does not seem to operate downstream of the Src family proteins either, because the amount of Thr202/Tyr204-phosphorylated ERK in cell lysates was unaltered in cells treated with LIF and SU6656, compared with cells treated with LIF alone (Fig. 2C).

The Src Family Is Required for ES Cell Self-renewal—To determine whether the Src family is necessary for maintaining ES cells in the undifferentiated state, mouse and human ES cells were treated with the selective kinase inhibitor SU6656 and cell differentiation was assessed. Mouse and human ES cells were cultured on MEFs for 6 days in ES media with 500 μM SU6656 and/or LIF and studied the phospho-Tyr705 STAT3 antibody (results not shown). Be - a dose-dependent decrease in the proportion of undifferentiated, AP-positive colonies in response to SU6656. The colonies exposed to SU6656 were also fewer and smaller, suggesting that the kinase inhibitor negatively affects cell growth (Fig. 3, E–G). The reduction in colony size due to SU6656 addition was found to result from diminished ES cell growth, as assessed by cell counting (results not shown). Mouse ES cells were cultured in gelatin-coated dishes for 6 days in increasing amounts of LIF (Fig. 3A). In accord with previous reports, LIF was required for mES cell self-renewal; ~80% of the colonies were undifferentiated when cultured in 125 pM LIF, whereas less than 5% of the colonies were completely undifferentiated in the absence of LIF (Fig. 3A).

Oct3/4 expression was analyzed in mES cells cultured for 8 days in various concentrations of LIF or with 500 μM LIF and increasing levels of SU6656. Although the decrease in Oct3/4 expression in response to differentiation is slower and less robust than that observed for alkaline phosphatase staining, immunohistochemistry and subsequent analysis by flow cytometry for Oct3/4 revealed a dose-dependent decrease in the number of Oct3/4+ cells in response to SU6656 (Fig. 3D), a result consistent with the notion that inhibition of Src family kinases leads to ES cell differentiation. Other genes expressed in undifferentiated ES cells, such as Nanog and FGF4 (12, 13, 28), were also decreased in SU6656-treated mES cells, as determined by semi-quantitative RT-PCR (Fig. 3K). Furthermore, Src family inhibition increased the expression of GCNF, a gene that represses oct3/4 gene activities in differentiating cells (29).
Samples not treated with reverse transcriptase were run in parallel to control for contaminating genomic DNA. These controls did not yield any detectable PCR product (results not shown).

To assess the mechanisms by which SU6656 inhibits ES cell growth, mouse and human ES cells were cultured in increasing concentrations of SU6656 inhibitor for 2 days, after which cell death (Fig. 3L) and BrdUrd incorporation (Fig. 3M) was determined. Src family inhibition caused a moderate, dose-dependent decrease in DNA synthesis and cell viability. A change in proliferation of ES cells may be related to a move from the undifferentiated state, because retinoic acid (RA) induces similar effects on ES cell growth (results not shown). Thus, we conclude that the loss of self-
renewal by Src family inhibition is not a consequence of massive cell death or a complete block in the cell cycle, but rather it is due to a combined partial effect upon both of these occurring in conjunction with differentiation.

Expression of yes siRNA Induces Differentiation and Reduces the Ability to Generate Stable ES Cell Clones—To specifically establish the role of the Src family member cYes in self-renewal of mES cells, down-regulation of cYes gene expression was achieved by RNA interference. Two different siRNA constructs for yes were designed and inserted immediately downstream of the U6 polymerase III promoter of the pSilencer™ vector. siRNA for EGFP and siRNA for nanog were used as negative and positive controls, respectively.

Transient transfection of mES cells with a mixture of plasmids including puro-EGFP-expressing EGFP linked to a puromycin-resistance gene, a vector expressing RFP, and empty pSilencer™ plasmid expressing non-sense siRNA (top), or with puro-EGFP or RFP plus pSilencer™ plasmids expressing EGFP siRNA (bottom); cells were visualized in a fluorescent microscope. Note that EGFP siRNA decreases the GFP signal (bottom left) but leaves the RFP signal intact (bottom middle). B, mouse ES cells were transfected with puro-EGFP or with puro-EGFP and pSilencer™ plasmids expressing nanog siRNA. Two days after transfection, cells were subjected to RT-PCR for Nanog and β-actin. C, mouse ES cells were transfected with puro-EGFP or with puro-EGFP and pSilencer™ plasmids expressing two different yes siRNA (yes1 and yes2). After 3 days, the GFP-positive cells were sorted by flow cytometry and subjected to RT-PCR for cYes, Nanog, GCNF, and β-actin. Densitometric scannings were performed, and the fold changes in gene expression compared with untreated cells and normalized to β-actin were calculated (B and C). D, mouse ES cells were transfected with pSilencer™ plasmids expressing EGFP (EGFP-control), Yes1, Yes2, or nanog siRNA under the control of the U6 RNA promoter together with puro-EGFP. Cells were then cultured for 14 days with 3 μg/ml puromycin, and the number of colonies was counted.

**Fig. 4.** yes siRNA induces differentiation and reduces the efficiency of making stable clones. A, EGFP reduces the expression of GFP but not RFP. mES cells were transfected with a mixture of plasmids including puro-EGFP-expressing EGFP linked to a puromycin-resistance gene, a vector expressing RFP, and empty pSilencer™ plasmid expressing non-sense siRNA (top), or with puro-EGFP or RFP plus pSilencer™ plasmids expressing EGFP siRNA (bottom); cells were visualized in a fluorescent microscope. Note that EGFP siRNA decreases the GFP signal (bottom left) but leaves the RFP signal intact (bottom middle).
SU6656 and RA exhibit synergistic effects upon mES cell differentiation. Cells were cultured in ES media alone (A, F, and K1), 100 nM RA (B, G, and K2), 0.1 nM RA (C, H, and K3), 1 μM SU6656 (D, I, and K4), 100 nM RA + 1 μM SU6656 (K5), or 0.1 nM + 1 μM SU6656 (E, J, and K6). After 8 days, cells were immunostained with β-tubulin (red) and α-1-fetoprotein (green) to study the extent of the differentiation (B–J). Note that the mES cells that have been cultured in 100 nM RA (B and G), or with 0.1 nM RA together with 1 μM SU6656 (E and J), show a similar...
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colonies were picked and analyzed for cYes expression but none exhibited reduced cYes protein levels. This result suggests that cells exhibiting reduced or abolished cYes or nanog expression are unable to form colonies (results not shown).

Synergistic Effects of Retinoic Acid and Src Family Inhibition on mES Cell Differentiation—To further examine the role of the Src family in ES cell differentiation, we cultured mES cells in the absence or presence of RA and/or SU6656. Culture in 1 μM SU6656 had no demonstrable effect upon differentiation (Fig. 5, D, I, and K), nor did low levels of RA (0.1 nM; Fig. 5, C, H, and K). A thousand times higher level of RA (100 nM), on the other hand, caused a significant increase in differentiation when added alone or together with SU6656 (Fig. 5, H, and K). Interestingly, a combination of 1 μM SU6656 and 0.1 nM RA mediated a dramatic and synergistic increase in multilineage differentiation (Fig. 5, E, J, and K). Differentiation was confirmed by antibody staining for neuronal-specific class III β-tubulin, and AFP, which identifies endodermal/liver cells (Fig. 5, A–J). Moreover, semi-quantitative RT-PCR was used to detect genes that define undifferentiated cells such as Cripto (15, 30), FGF4, and Nanog, as well as genes that are up-regulated in differentiated cells, including cardiac actin (c-actin) and Disabled-2 (Dab2; Ref. 31). Mouse ES cells treated with 0.1 nM RA together with 1 μM SU6656 expressed lower levels of Cripto and FGF4 mRNA, and higher levels of Dab2 and c-actin message compared with mES cells cultured in 0.1 nM RA or 1 μM SU6656 alone (Fig. 5K). The semi-quantitative RT-PCR results for Nanog, Cripto, FGF4, Dab2, and β-actin were confirmed by quantitative real-time PCR (results not shown). From these data we conclude that Src family inhibition and low levels of RA work in synergy to induce differentiation of mES cells.

DISCUSSION

In the present study we demonstrate that the Src family member cYes is highly expressed in mouse and human ES cells, and that its activity is regulated by LIF and serum. Moreover, we show that cYes kinase activity is down-regulated when cells differentiate. Activation of Hck shows similar results in response to LIF, although the protein levels of Hck declined when mES cells differentiated, and the activation of Hck in response to LIF was quicker and more transient than for cYes (Ref. 20 and results not shown). Moreover, selective inhibition of the Src family increases ES cell differentiation, suggesting that one or more of its members are required for self-renewal of undifferentiated ES cells.

Previous studies on genes and signaling pathways that contribute to the maintenance of the undifferentiated state have mainly been performed in mouse ES cells. Therefore, little is known about the genes that may regulate the self-renewal and pluripotency of hES cells. Mouse and human ES cells may, however, share some signaling pathways that regulate self-renewal. We propose that one such pathway involves Src kinases, because chemical suppression of this kinase family in mouse and human ES cells results in the loss of alkaline phosphatase expression and reduced cell proliferation. cYes kinase activity is down-regulated when human and mouse ES cells differentiate into EBs, suggesting that cYes may be a particularly important Src family member in both human and mouse ES cells. Because both serum and LIF can activate cYes in mES cells, one or more factors in serum might work in concert with LIF to activate cYes. Similarly, factors expressed by the MEFs or present in serum replacement might activate cYes in hES cells, although this remains to be shown. In this regard, it will be interesting to see if bone morphogenetic proteins regulate Src family and cYes kinase activity.

The ability of vYes and vSrc to induce cellular transformation suggests that their cellular counterparts may be involved in the control of cell proliferation and cell shape associated with the transformed phenotype. Indeed, the present data indicate that one important function of the Src family in ES cells is to support cell growth. This role might be best illustrated by the finding that inhibition of Src family kinases induced a decrease in cell growth. The SU6656 inhibitor used in this study did not seem to induce nonspecific toxicity at levels below 4 μM. Not only did the cells look healthy, but they also continued to proliferate, albeit at a lower rate, and could be induced to differentiate into several different cell types when added together with RA (see discussion below).

The present study suggests that the Src family plays an important role in maintaining the undifferentiated state of ES cells because the SU6656 kinase inhibitor seemed to differentiate the ES cells. This was demonstrated by the cells acquiring a fibroblast-like phenotype, in contrast to the small and evenly shaped undifferentiated mES cells. The inhibitor induced other signs indicative of differentiation, such as reduced levels of AP, Oct3/4, Nanog, and FGF4, and increased levels of GCNF. To study the role of cYes for ES cell self-renewal, it was necessary to specifically reduce its expression or activity in mES cells. By introducing siRNAs specific for cYes in mES cells, we could transiently reduce its expression but were unable to generate stable clones. Thus, when mES cells were transfected with yes siRNA and a vector containing a puromycin-resistant gene (puro-GFP) and selected with puromycin, we obtained fewer colonies compared with the cells transfected with puro-GFP alone, but none of the colonies exhibited reduced levels of yes. Introducing siRNA for the mES cell pluripotency gene nanog induced similar effects. A possible interpretation of these results is that the siRNA for yes and nanog affect cell growth and survival, preventing the siRNA-expressing cells from forming colonies. The other possibility is that the siRNA increases the propensity of mES cells to differentiate and thereby increases their sensitivity to puromycin. Evidence from transient transfections suggests that the mES cells expressing yes siRNA are indeed differentiating. The yes siRNA induced a reduction in the mRNA levels of nanog, whereas it increased the levels of GCNF. GCNF, an orphan nuclear receptor, represses oct3/4 gene expression and is induced by RA (29). Nanog has been proven to be required for the maintenance of undifferentiated ES cells, is expressed in undifferentiated ES cells and is down-regulated when cells differentiate (12, 13). The observations that yes siRNA induces changes in the expression of nanog and GCNF mRNA expression and in colony formation could imply that yes exhibits a significant and unique function in mES cells despite the presence of other Src family members. This is somewhat surprising, because Src family members, in particular, cSrc, Fyn, and cYes, have been shown to be able to compensate for each other in knockout mice. Although cSrc and cYes are activated in response to many of the same signals and
may act redundantly, there are also several studies demonstrating cYes-specific signaling pathways (32–34). The siRNA results cannot, however, exclude the possibility that the absolute level of Src family activity is important for ES cell self-renewal and that other members of this family play similar roles in ES cells.

We also show that SU6656 and low levels of RA induced a synergistic differentiation of mES cells. Inhibition of the Src family may put ES cells in a state that is more sensitive or susceptible to differentiation signals by agents such as RA. In this regard, we note that the mRNA levels of nuclear receptor GCNF are increased in response to both RA and SU6656 independently (Fig. 3K, and Ref. 29). Thus, the role of the Src family may be to maintain expression of genes that keep ES undifferentiated, thereby obstructing their ability to differentiate.

It has previously been hypothesized that Src family members may signal through the Ras/MAPK pathway (22, 35). Moreover, stimulation of STAT3 activity by EGF, interleukin-6, and hepatocyte growth factor in lung carcinoma cells has been reported to require activity of Src family members (27). The present study, however, shows that inhibition of Src family kinase activity by SU6656 does not interfere with the LIF-induced phosphorylation of JAK1/STAT3 or Ras/MAPK. This result suggests that the Src family is not a major activator of these pathways in mES cells. Because cYes and Hck can be activated by LIF, we suggest that the Src family rather represents a third, independent pathway downstream of LIF in mES cells.

Although the present results on cYes are relevant to mechanisms maintaining ES cell lines in vitro, they may not be relevant to the inner cell mass in embryos because yes knockout mice are viable and fertile (36). cYes is not the only gene that is important for ES self-renewal, but it seems less significant for keeping pluripotent cell populations in vivo. For instance, although the LIF/STAT3 pathway plays a significant role in maintaining self-renewal of mES cells, it has been reported that lif (37, 38), lifrβ (39), gpt130 (39), and stats3 (40) knockout mice can develop beyond the egg cylinder stage.

In conclusion, the results presented in this paper suggest that the Src family kinases are important for maintaining growth and self-renewal in both human and mouse ES cells, and that cYes may be a particularly important member of this family. Future work to establish the signaling pathways that operate downstream of cYes and other Src family kinases should generate more information about the molecular features that underlie the maintenance and propagation of pluripotent ES cells.

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