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HAL Id: hal-02667518
https://hal.inrae.fr/hal-02667518v1

Submitted on 31 May 2020

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This information is current as of August 28, 2007

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Self-renewal of Murine Embryonic Stem (ES) Cells is Supported by the Serine/Threonine Kinases Pim-1 And Pim-3

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ABSTRACT

Pim-1 and *pim-3* encode serine/threonine kinases involved in the regulation of cell proliferation and apoptosis in response to cytokine stimulation. We analysed the regulation of *pim-1* and *pim-3* by the Leukemia Inhibitory Factor (LIF)/gp130/Signal Transducer and Activator of Transcription (STAT)-3 pathway and the role of Pim-1 and Pim-3 kinases in mouse Embryonic Stem (ES) cell self-renewal. Making use of ES cells expressing a G-CSF:gp130 chimaeric receptor and a hormone-dependent STAT3-ER^{T2}, we showed that expression of *pim-1* and *pim-3* was up-regulated by LIF/gp130-dependent signaling and the STAT3 transcription factor. ES cells overexpressing *pim-1* and *pim-3* had a greater capacity to self-renew and displayed a greater resistance to LIF starvation based on a clonal assay. In contrast, knock-down of *pim-1* and *pim-3* increased the rate of spontaneous differentiation in a self-renewal assay. Knock-down of *pim-1* and *pim-3* were also detrimental to the growth of undifferentiated ES cell colonies and increased the rate of apoptosis. These findings provide a novel role of Pim-1 and Pim-3 kinases in the control of self-renewal of ES cells.

INTRODUCTION

The propagation of mouse embryonic stem (ES) cells is dependent on the presence of leukemia inhibitory factor (LIF) which engages a heterodimeric receptor complex consisting of LIF receptor (LIFR) and gp130. This complex activates the Janus-associated tyrosine kinases (JAK), which phosphorylate the receptor chains. The phosphorylated tyrosines then act as docking sites for the signal transducer and activator of transcription STAT3 [1]. Phosphorylation of STAT3 by JAKs, followed by its dimerization and nuclear translocation, plays a crucial role in self-renewal of ES cells [2]. Hence, expression of a dominant negative STAT3 mutant in ES cells forces differentiation [3], whereas expression of a hormone-dependent STAT3 mutant that can be activated directly by estradiol sustains self-renewal without the addition of LIF [4]. Activation of the LIFR:gp130 receptor also induces JAK-dependent tyrosine phosphorylation of the SHP-2 phosphatase. However, SHP-2 recruitment opposes STAT3-dependent self-renewal and facilitates differentiation by promoting the activation of the Ras/Mitogen-Activated Protein Kinase (MAPK) pathway [5].

A number of genes whose expression is activated by LIF have been identified in ES cells [4,6-9] although only two, *c-myc* and *Krüppel-like factor (klf)4*, have been shown to contribute to the inhibition of differentiation. *c-myc* is a target gene of the LIF/STAT3 pathway. Forced expression of a hormone-dependent Myc-ER temporarily inhibits the differentiation of ES cells induced by withdrawal of LIF [8]. Similarly, forced expression of Klf-4 delays differentiation induced by LIF deprivation [9].

The *pim* genes encode the serine/threonine kinases Pim-1, Pim-2 and Pim-3, involved in the regulation of cell growth and apoptosis [10]. Pim-1 and Pim-2 each have the ability to cooperate with Myc in experimental T-cell lymphomagenesis [11,12], a finding which established Pim-1 and Pim-2 as proto-oncogenes and important players in the process of malignant transformation. Pim kinases contributes to the regulation of cell proliferation in response to stimulation by cytokines and growth factors. The expression of the *pim-1* gene can be induced *via* the activation of STAT3 and STAT5 which bind directly to the *pim-1* promoter in eosinophils [13]. In lymphoid cells, *pim-1* is activated by Interleukin-6 *via* STAT3 and regulates proliferation in cooperation with Myc [14]. All three Pim kinases also play an important role in the prevention of cell death [15,16].

In this paper, we studied the regulation of the *pim-1* and *pim-3* genes by the LIF/STAT3 pathway, as well as their role in the control of proliferation and differentiation, in ES cells. The data presented show that Pim-1 and Pim-3 play an important role in maintaining the ES cell identity by inhibiting differentiation and apoptosis.

MATERIAL AND METHODS

Plasmid construction. The hormone dependent STAT3-ER^{T2} transcription factor was generated by fusing the coding sequence of the mouse STAT3 transcription factor to a 5'-XhoI/EcoRI-3' fragment containing the entire ER^{T2} domain [17]. The resulting cDNA was subcloned into the EcoRI site in *pPCAGIZ* [3] to generate *pPCAGIZ-STAT3-ER^{T2}*. Full length cDNA encoding mouse Pim-1 was subcloned into the blunted BstXI site in *pPHCAG* [3] and into the blunted XhoI site in *pPHPGK* [18] to generate *pPHCAG-Pim1* and *pPHPGK-Pim1*, respectively. Full length cDNA encoding mouse Pim-3 was subcloned into the blunted EcoRI site in *pPCAGIZ* [3] and into the XhoI site in *pPHPGK* to generate *pPCAGIZ-Pim3* and *pPHPGK-Pim3*, respectively.

The BLOCK-iT lentiviral RNAi Gateway® vector kit (Invitrogen, Ref. K4943-00) was used to generate lentiviral vectors expressing small hairpin (sh)RNA directed to *GFP*, *Pim-1* and *Pim-3*, according to manufacturer's instructions. In the *pLenti6/BLOCK-iT* backbone vector, the blunted KpnI (blunt)/XhoI fragment was replaced by a 1.3 Kb ClaI (blunt)/XhoI fragment containing the *PGK* promotor and the *neo^r* selectable gene. Oligonucleotides encoding shRNAs for *GFP*, *Pim-1*, *Pim-3* and *Stat3* were subcloned into the resulting *pLenti6/BLOCK-iT-PGKneo* vector to generate *pLenti6/BLOCK-iT-PGKneo-GFP*, *pLenti6/BLOCK-iT-PGKneo-Pim1*, *pLenti6/BLOCK-iT-PGKneo-Pim3* and *pLenti6/BLOCK-iT-PGKneo-Stat3*, respectively. The sequences of shRNA are as follows: GFP: 5'-GCCACA A CGTCTATATCATTTCAAGAGAATGATAT AGACGTTGTGGC-3'; Pim-1: 5'-GCAAGACCTCTTCGACTTTATTTCAAGA GAATAAAGTCGAAGAGGTCTT GC-3'; Pim-3: 5'-CACGGTCTACACTGACTTTGATGTTCAA GAGACATCAAAGTCAG GTAGACCGTG-3'; STAT3: 5'GGAGCTGTTCAGAAACTTATTCAAGA GATAAGTTTC TGAACAGCTCC-3'.

ES cell culture, electroporation and infection. All ES cell lines were routinely cultured in Glasgow's Modified Eagle's Medium (GMEM) supplemented with 10% fœtal calf serum and 1000 U/ml of LIF as described previously [19]. To induce differentiation, cells were allowed to aggregate in hanging drops in ES cell medium without

LIF [20]. After 2 days, embryoid bodies were collected and further grown in suspension for 1 to 4 days. For episomal supertransfection, E14/T cells [18] were electroporated with 20 µg of supercoiled vectors at 200 V and 960 µF in a 0.4 cm cuvette. Cells were plated at 5.10^4 cells per 10 cm dish, cultured in the presence of 100 µg/ml of hygromycin B (Roche) or of 1 µg/ml of zeocine (Cayla) for 7 days. Resistant colonies were pooled and further propagated in selection medium for 8 to 10 days prior to analysis.

Lentiviral vectors expressing shRNA were produced using the BLOCK-iT lentiviral RNAi expression system (Invitrogen, Ref. K4944-00) according to the manufacturer's instructions. For lentiviral infection, CGR8 were plated at a density of 10^4 cells in 24-well plates in 1 ml of medium composed of 100 µl of ES cell medium and 900 µl of culture supernatant from virus-producer cells. After 48 hrs, ES cells were trypsinized, replated at 10^4 cells per gelatin-coated 10 mm tissue culture dish and further cultured in complete ES cell medium supplemented with 250 µg/ml of G418 for 6 days.

Stimulation with LIF, G-CSF and tamoxifen, cell lysates and immunoblotting. ES cells were plated at a density of 2×10^6 cells per 10 cm dish and cultured for 24 hrs in medium lacking LIF. The following day, cells were stimulated with 10,000 U/ml of LIF, 30 ng/ml of G-CSF (Calbiochem) or 100 nM of 4'hydroxy-tamoxifen (4'-OHT) (Calbiochem) for the time indicated. Cells were then washed and scrapped off with ice-cold PBS, centrifugated and freezed at - 80°C. Cell pellets were lysed in 20 mM Hepes pH 7.4, 100 mM NaCl, 50 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and cocktail of protease inhibitor (Roche) for 1 hr at 4°C. Protein lysates were then cleared by centrifugation (14,000 RPM for 20 min). For immunoblotting, 30 µg of total proteins were resolved by SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. After overnight treatment with blocking buffer (50

mM Tris-Hcl pH 7.6, 150 mM NaCl, 5% dry milk), the membranes were probed with specific monoclonal or polyclonal antibodies [Santa Cruz: anti-Pim1 (Santa Cruz, sc-13513) and anti-Cdc25A (Santa Cruz, sc-7389). Blots were incubated with horseradish peroxidase-coupled anti-mouse, anti-rabbit, or anti-goat IgG and developed using ECL reagents (Amersham).

Semi-quantitative and real-time PCR. RNA was extracted using RNAeasy kits with on-colomn DNAse digestion and reverse transcription was carried out with Omniscript, according to the manufacturer's recommendations (Qiagen). PCR reactions were performed with a Perkin-Elmer thermal cycler, operating on a regimen of 96°C for 5 sec, 55 to 57°C (according to primers) for 15 sec and 72°C for 60 sec, for 35 cycles, followed by 72°C for 10 min. Primers and annealing temperatures used to detect *Nanog*, *Oct-4*, *pim-1*, *pim-2, pim-3*, *cdc25A, rex-1* and *ß-actin* by RT-PCR are as follows:

- *Nanog*: cDNA length, 438 bp; annealing temperature, 55°C; 5' primer (5' to 3'), TACCTCAGCCTCCAGCAGA; 3' primer (5' to 3'), CCTCCAAGTCACTGGCAG;

- *Oct-4*: cDNA length, 974 bp; annealing temperature, 56°C; 5' primer (5' to 3'), ATGGATCCTCGAACCTGGC; 3' primer (5' to 3'), TCAGTTTGAATGCATGCATGGGAG.

- *pim-1*: cDNA length, 231 bp; annealing temperature, 55°C; 5' primer (5' to 3'), GCCCTCCTTTGAAGAAATCC; 3' primer (5' to 3'), GGACCTGGAGTCTGGAATGA.

- *Pim-2*: cDNA length, 212 bp; annealing temperature, 55°C; 5' primer (5' to 3'), ACATGGTCTGTGGGGACATT; 3' primer (5' to 3'), TCCTTTGGAGGAGTTGATGG.

- *pim-3*: cDNA length, 167 bp; annealing temperature, 57°C; 5' primer (5' to 3'), AGCAGTGACCTCTGACCCCT; 3' primer (5' to 3'), TCAAGTATCCACCCAGGGCA.

- *Cdc25A*: cDNA length, 711 bp; annealing temperature, 52°C; 5' primer (5' to 3'), TCTGTCTAGATTCTCCTGG; 3' primer (5' to 3'), CAGATGCCATAATTTCTGGAG.

- *Rex-1*: cDNA length, 130 bp; annealing temperature, 55°C; 5' primer (5' to 3'), CGTGTAACATACACCATCCG; 3' primer (5' to 3'), GAAATCCTCTTCCAGAATGG.

- *ß-actin*: cDNA length, 200 bp; annealing temperature, 55°C; 5' primer (5' to 3'), TGAAACAACATACAATTCCATCATGAA GTGTGA; 3' primer (5' to 3'), AGGAGCGAT AATCTTGATCTTCATGGTGCT.

Quantitative PCR was performed using the LightCycler^{TM} 1.5 system and the LightCycler Fast Start DNA Master SYBR Green I kit (Roche Applied Science) according to the manufacturer's instructions. Reactions were carried out in a total volume of 20 µl, comprising 0.4 µM of each primer, 3.5 mM MgCl$_2$, 2 µl SYBR Green, and 2 µl of diluted cDNA. Amplification and online monitoring was performed using the LightCycler^{TM} 1.5 system (Roche Applied Science). Following 40 amplification cycles, melt-curve analyses were performed to verify only the desired PCR product had been amplified. PCR efficiency of both the target and reference genes was calculated from the derived slopes of standard curves by the LightCycler software (Roche Molecular Biochemicals LightCycler Software, Version 4.0). These PCR efficiency values were used to calculate the relative quantification values for calibrator-normalised target gene expression by the LightCycler relative quantification software (Version 4.0). In all cases target genes were normalised to *ß-actin*. A *ß-actin* cDNA was used as normalization control to ensure constant *ß-actin* levels in all samples regardless experimental conditions.

Detection of alkaline phosphatase (AP) activity. Dishes were fixed in methanol for 15 min, then stained for 15 min with a solution containing 1 mg/ml Fast Red TR salt^{TM} (Sigma) dissolved in 0.1 M Tris, pH 9.2, containing 200 mg/ml naphtol AS-MX phosphate [21, 22].

Detection of Stem cell Specific Embryonic Antigen (SSEA)-1 expression. ES cells were trypsinized and the cell suspension incubated in culture medium for 6 hrs on a rocker platform. Cells were subsequently Fc-blocked by treatment with 1 µg of human IgG/10^5 cells for 15 min at room temperature. Then, 10^5 cells in 25 µl of PBS supplemented with 0.5% BSA were incubated for 30-45 min at 4°C with 50 µl (50µg/ml) of the Phycoerythrin-conjugated mouse monoclonal anti-SSEA1 (R&D Systems, Ref. FAB2155P). Cells were washed twice with PBS. Fluorescence intensity was determined by flow cytometry on a FACScan (BD FACS CANTO II), and data acquisition was performed using the BD FACS Diva software.

TUNEL assay. The ApopTag® Red In Situ Apoptosis Detection Kit (Abcus, Ref. S7165) was used to identify apoptotic cells. All reactions were performed according to the manufacturer's instructions.

<div style="text-align:center">

RESULTS

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Influence of LIF on the transcriptional regulation of *pim-1* and *pim-3*.
Expression of *pim-1*, *pim-2*, and *pim-3*, was examined by semi-quantitative RT-PCR in undifferentiated CGR8 cells and in 1-6 day-old embryoid bodies (Figure 1 A). ES cells expressed elevated levels of *pim-1* and *pim-3* transcripts, which both declined abruptly during the first day of differentiation. By contrast, *pim-2* transcript level increased during the first two days of differentiation and dropped from third day onwards. Thus, *pim-1* and *pim-3* exhibit a unique expression pattern characterized by a drop in trancript levels within 24 hours following withdrawal of LIF. Down-regulation of *pim-1* and *pim-3* expression takes place prior to the loss of pluripotency marker *Oct-4* [23] occuring between day 3 and 4.

To examine the role of LIF in the regulation of *pim-1* and *pim-3* expression, CGR8 ES cells

were LIF-deprived for 24 hrs, then restimulated with 10.000 U/ml of LIF for 1 hr. Withdrawal of LIF for 24 hrs did not induce loss of ES cell pluripotency (see Supplementary data 1). This stimulation protocol led to a strong activation of the LIF signalling pathway revealed by a 14-fold increase in the mRNA level of the LIF/STAT3 target gene *junB* [4,6] (data not shown). *Pim-1* and *pim-3* transcript levels increased 1.3 +/- 0.1-fold (p < 0.06) and 1.74 +/- 0.13-fold (p < 0.05) respectively, after LIF stimulation (Figure 1 B, left panel). Activation of *pim-1* and *pim-3* after stimulation by LIF was also observed in E14/T ES cells [*pim-1:* 2.4 +/- 0.2 fold, p < 0.001; *pim-3*: 1.35 +/- 0.05 fold, p < 0.01] (Figure 1 B, right panel). The level of *Pim-1* expression also increased at the protein level in response to LIF stimulation (2.1 +/- 0.4-fold, p < 0.01). In contrast, the levels of Cdc25A phosphatase, a substrate of Pim-1 [24], remained unchanged (Figure 1C). Thus, the levels of *pim-1* and *pim-3* mRNA and the level of Pim-1 kinase were up-regulated as a consequence of LIF receptor stimulation in ES cells.

Contribution of STAT3 to the transcriptional regulation of *pim-1* and *pim-3.*

We made use of engineered ES cell lines stably expressing GRgp278, GRgp(Y118F), and GRgp(Y126-275F) receptors [3, 5] to study the role of STAT3 in the regulation of *pim-1* and *pim-3* expression. GRgp278 is a chimaeric receptor consisting of the extracellular domain of the G-CSF receptor fused to the transmembrane and cytoplasmic region of gp130. Homodimerization of the GRgp278 receptor, mediated by G-CSF, induces recruitments of both STAT3 and SHP-2 and promotes self-renewal. GRgp(Y118F) is a mutated chimaeric receptor in which phenylalanine has been substituted for tyrosine 118 in the intracytoplasmic domain, which prevents the recruitement of SHP-2 by the activated receptor. In the GRgp(Y126-275F) receptor, phenylalanines have been substituted for tyrosine 126 and 275 which strongly reduces recruitement of STAT3 by the activated receptor (see Supplementary data 2).

ES cells expressing wild-type, SHP2-binding deficient, and STAT3-binding deficient chimaeric receptors were LIF-deprived for 24 hrs and restimulated for 1 hr, either with 10,000 U/ml of LIF to activate the endogenous gp130 receptor, or with 30 ng/ml of G-CSF to activate the chimaeric receptor. Stimulation of GRgp278 (wild-type) with G-CSF resulted in increased levels of *pim-1* (1.6 +/- 0.2-fold, p < 0.01) and *pim-3* (2.8 +/- 0.4-fold, p < 0.001) mRNAs (Figure 2 A,C). Similarly, stimulation of the GRgp(Y118F) cells with G-CSF resulted in increased levels of *pim-1* (1.5 +/- 0.25-fold, p < 0.05) and *pim-3* (3.35 +/- 0.45-fold, p < 0.001) mRNAs, indicating that recruitment of SHP-2 to gp130 receptor is not required for the transcriptional activation of *pim-1* and *pim-3* genes. In the STAT3-binding deficient cell line [GRgp(Y126-275F)], LIF and G-CSF were equally efficient at activating the expression of *pim-1* indicating that recruitment of STAT3 to the activated gp130 receptor is not essential to the transcriptional activation of *pim-1*. Examination of protein levels confirmed the activation of Pim-1 expression in response to LIF and G-CSF stimulation in the cell lines expressing the wild-type, SHP2- and STAT3-binding deficient receptors (Figure 2 B). By contrast, G-CSF failed to activate the expression of *pim-3* in the GRgp(Y126-275F) indicating that activation of STAT3 is required to the transcriptional activation of *pim-3* (Figure 2 C).

Pim-1 is a target gene of STAT3 which contributes to Interleukin-6-dependent regulation of proliferation in lymphoid cells [14]. This prompted us to examine further if STAT3 activity could regulate *pim-1* expression in the absence of gp130 receptor activation. To this aim, a conditional mutant of STAT3 was generated by fusing the STAT3 coding sequence to ER^{T2}, a mutated ligand binding domain of the human oestrogen receptor [17, 25]. The STAT3-ER^{T2} fusion protein was overexpressed by means of supertransfection of the *pPCAGIZ-STAT3-ER^{T2}* episomal expression vector into the E14/T cell line in the presence of 4'-hydroxy-tamoxifen (4'OHT) [18]. *pPCAGIZ-STAT3-ER^{T2}* transfectants formed a uniform population of

undifferentiated ES cells which was undistinghuishable from the sister population of *pPCAGIZ-STAT3-ER^{T2}* transfectants propagated in the presence of LIF (data not shown). To assess the regulation of *pim-1* and *pim-3* by STAT3 activity, STAT3-ER^{T2}-transfectants were deprived of 4'OHT for 24 hrs then restimulated with 100 nM 4'OHT for 2 hrs (Figure 3 A). This resulted in increased levels of *pim-1* (1.5 +/- 0.1 fold, $p < 0.01$) and *pim-3* (2.0 +/- 0.1 fold, $p < 0.001$) mRNAs. Stimulation by 4'OHT in the presence of cycloheximide led to a 1.7-fold increase in mRNA levels, indicating that 4'OHT-induced transcriptional activation of *pim-1* and *pim-3* was not dependent on *de novo* protein synthesis. The observed effect is STAT3-specific since E14/T ES cells expressing a hormone-dependent Cre-ER^{T2} showed no increase in *pim-1* and *pim-3* expression following stimulation with 4'OHT. The increase of *Pim-1* mRNA level observed in response to 4'OHT was paralleled by a 2.5-fold increase in protein level (Figure 3 B).

Together, these data indicate that the expression of *pim-1* and *pim-3* genes is regulated by STAT3 activity in ES cells.

Contribution of Pim-1 and Pim-3 kinases to the inhibition of ES cell differentiation.

The role of Pim kinases in the inhibition of ES cell differentiation was examined by RNA interference, making use of lentiviral vectors encoding small hairpin interfering RNA (shRNA) for *pim-1* (*pLenti6/BLOCK-iT-PGKneo-Pim1*), *pim-3* (*pLenti6/BLOCK-iT-PGKneo-Pim3*) and *GFP* (*pLenti6/BLOCK-iT-PGKneo-GFP*) and the *neo^r* selectable gene. Fourty Eight hrs after infection, CGR8 ES cells were replated at clonal density and further cultured in selection medium for 7 days to kill non infected cells (approximately 50% of the population). The percentages of undifferentiated (AP^+), mixed (AP^+/AP^-) and differentiated (AP^-) G418-resistant colonies were calculated (Figure 4 A). CGR8 ES cells expressing the shGFP maintained undifferentiated AP^+ colonies at between 71 and 76 % in this assay. Expression of shPim-1 or shPim-3 reduced, albeit moderately, the proportion of undifferentiated colonies compared to control (shPim-1, - 20%, $p < 0.01$; shPim-3, - 27.9%, $p < 0.001$). The decrease in percentage of undifferentiated AP^+ colonies observed in ES cells expressing shPim-1 and shPim-3 were paralleled by an increase in the percentage of mixed AP^+/AP^- colonies (shPim-1, + 17.3%, $p < 0.01$; shPim-3, + 22.3%, $p < 0.001$) as well as of AP^-, flat, differentiated colonies (shPim-1, + 2.6%, $p < 0.05$; shPim-3, + 5.5%, $p < 0.01$). Expression of *rex-1*, a marker of pluripotency [26], was analysed by quantitative RT-PCR and showed respectively, 25% and 49% of reduction in transcripts levels in cells expressing shPim-1 and shPim-3, compared to cells expressing shGFP (Figure 4 B). Cells expressing shSTAT3, a shRNA directed to STAT3, showed a 90% reduction in *rex-1* transcripts levels.

Gene expression analysis by real-time PCR showed that the levels of *pim-1* and *pim-3* transcripts were reduced only 2-fold in the infected cell population (data not shown), possibly explaining the relatively small differences observed in the proportions of undifferentiated *versus* mixed/differentiated colonies in the self-renewal assay. CGR8 cells expressing shGFP, shPim-1 and shPim-3 were subcloned and the level of expression of *pim-1* and *pim-3* analysed to select clones showing the highest interference (Figure 4 C). Clones 1, 2, and 7 (shPim-1), and clones 1, 3, and 5 (shPim-3) - all six showing a residual expression of *pim-1* and *pim-3* between 20% and 38% with respect to levels measured in the shGFP control - were selected for further analysis. All shPim-1 and shPim-3 clones formed from 3.3 to 16-fold less AP^+ undifferentiated colonies and 3.2 to 4-fold more AP^- differentiated colonies than the shGFP clone in the self-renewal assay (Figure 4 D). The stronger the interference of *pim-1* and *pim-3* expression is, the lower the percentage of undifferentiated colonies. Undifferentiated, mixed, and differentiated colonies were pooled and subsequently analysed for the expression of pluripotency markers. All shPim-1 and shPim-3 clones displayed reduced levels of transcripts encoding Oct-4 (50-66% reduction) and Nanog

(44-73% reduction) (Figure 4 E). They also displayed lower percentages of cells expressing the SSEA-1 antigen, a marker of pluripotent stem cells (shPim-1: 32-50%; shPim-3: 25-40%) compared to control cells (shGFP: 80%, $p < 0.001$) (Figure 4 F). In routine culture, all shPim-1 and shPim-3 clones displayed higher rates of spontaneous differentiation when compared to the shGFP control. Interestingly, interference in the shPim-1 and shPim-3 clones was progressively lost during serial passagings, indicating that ES cells with low Pim-1 and Pim-3 content are counter-selected (data not shown).

These results demonstrate that expression of Pim-1 and Pim-3 kinases are required to inhibit differentiation of ES cells.

Overexpression of Pim-1 and Pim-3 kinases delays LIF-induced differentiation.
Given that Pim-1 and Pim-3 kinases are necessary for self-renewal of ES cells, we asked if overexpression of Pim-1 and Pim-3 would allow them to overcome spontaneous differentiation induced by LIF starvation. Pim-1 and Pim-3 kinases were overexpressed by means of supertransfection of *pPHPGK-Pim1* and *pPHPGK-Pim3* episomal expression vectors into the E14/T cell line [18]. Engineered ES cells were plated at clonal density and further cultured for 7 days in the presence of LIF. The percentages of undifferentiated, mixed, and differentiated colonies were calculated (Figure 5 A). ES cells overexpressing Pim-1 and Pim-3 formed significantly more undifferentiated colonies (Pim-1, + 5.3%, $p = 0.05$; Pim-3, + 17.9%; $p < 0.001$), and significantly fewer mixed colonies (Pim-1, - 5.6%, $p < 0.05$; Pim-3, - 17.8%; $p < 0.001$), than control cells. These data indicate that overexpression of Pim-1 and Pim-3 reduces the rate of spontaneous differentiation in the presence of LIF.

In a second step, Pim-1 and Pim-3 transfectants were subjected to a clonal assay combined with a LIF rescue test to evaluate the recovery of ES cells following a short period of LIF starvation [27]. Cells were exposed to medium without LIF for 12, 24, 36 and 48 hrs and were

subsequently cultured for 5 to 7 days in medium supplemented with LIF. Then, the proportion of undifferentiated colonies was counted to assess the LIF rescue efficiency. In Pim-1 and Pim-3 transfectants, like in control cells, the proportion of undifferentiated colonies decreased increasingly with the duration of LIF deprivation, which indicates that overexpression of Pim-1 and Pim-3 is unable to fully sustain ES self-renewal in the absence of LIF (Figure 5 B). However, Pim-1 and Pim-3 transfectants showed a reduced sensitivity to LIF starvation. This was evidenced by a slower decrease in the proportion of undifferentiated colonies in ES cells overexpressing Pim-1 and Pim-3, compared to ES cells transfected with the empty vector (*pim-1 versus* control: $p < 0.001$; *pim-3 versus* control: $p < 0.05$). Together, these results are consistent with the overexpression Pim-1 and Pim-3 partially counterbalancing the effects of LIF deprivation on induction of differentiation.

Contribution of Pim-1 and Pim-3 kinases to the regulation of ES cell proliferation and apoptosis.
In the LIF-rescue experiment reported in Figure 5 B, we observed that the colonies generated from Pim-1 transfectants were larger than the control colonies (data not shown), suggesting that Pim-1 is also involved in the regulation of ES cell growth. We therefore asked if *pim-1* and *pim-3* knockdowns are detrimental to ES cell growth, making use of the ES cells expressing *pim-1-*, *pim-3-*, and *GFP*-specific shRNA. Because knock-down of *pim-1* and *pim-3* expression increases the rate of spontaneous differentiation, and ES cell differentiation is associated with increased cell-cycle duration [19], we thought to analyse the effect of *pim-1* and *pim-3* knock-down on the size of residual undifferentiated AP$^+$ colonies in the clonal assay. We observed that AP$^+$ colonies expressing the *pim-1* and *pim-3* shRNA were respectively 31% and 41% smaller than AP$^+$ colonies expressing the *GFP* shRNA ($p < 0.001$) (Figure 6 A). TUNEL analysis of the undifferentiated colonies showed a 28% (*pim-1*) and 35% (*pim-3*) increase in the number of positive cells (p <

7

0.05), indicating that knockdown of *pim-1* and *pim-3* increases the rate of apoptosis (Figure 6 B,C). Together, these results are consistent with *pim-1* and *pim-3* contributing to the regulation of ES cell growth by inhibiting apoptosis.

DISCUSSION

First, we have shown that the expression of *pim-1* and *pim-3* can be activated by LIF in two different ES cell lines. We also demonstrated that *pim-1* and *pim-3* expression can be activated by hormone-dependent STAT3 in the presence of protein synthesis inhibitor. Together, these findings establish *pim-1* and *pim-3* as novel target genes of STAT3 in ES cells. The *pim-1* promoter is known to contain a DNA sequence that potentially binds STAT oligomers [14]. Yet, ES cells expressing the STAT3-binding deficient G-CSG:gp130 chimaeric receptor partially activate the expression of *pim-1* in response to G-CSF. This observation suggests that, in the absence of STAT3 recruitment, another pathway can activate *pim-1*. Candidate pathways are the LIF/PI3K and LIF/Yes pathways which both contribute to inhibiting ES cell differentiation [22, 28]. Furthermore, STAT3 is not the only factor regulating *pim-1* and *pim-3* expression in ES cells. It was observed that deprivation of LIF for 24 hours reduces *pim-3* RNA level to 50% of its original level, and *pim-1* RNA level is not decreased by this treatment. This indicates that factors other than LIF and STAT3 contribute to the regulation of *pim-1* and *pim-3* expression. Loh *et. al.,* reported the mapping of Nanog binding sites in ES cells [29]. Mining their expression profiling data revealed that Nanog binding sites are present in the promoter of *pim-1*. Furthermore, we observed that *pim-1* and *pim-3* expression is down-regulated upon Nanog knock-down in CGR8 ES cells (unpublished data). Hence, the residual expression of *pim-1* and *pim-3* observed after withdrawal of LIF might result from a Nanog-dependent transcriptional activation mechanism.

Second, we demonstrated that overexpression of Pim-1 and Pim-3 decreases the rate of spontaneous differentiation in a clonal assay, enhances the resistance of ES cells to LIF starvation in a LIF rescue assay, whereas the knock-down of Pim-1 and Pim-3 increases the rate of spontaneous differentiation. These observations indicate that Pim-1 and Pim-3 kinases contribute to maintain ES cell pluripotency. Overexpression of Pim-1 and Pim-3 delayed the appearance of mixed and differentiated colonies in the LIF rescue assay, but failed to block differentiation in the absence of LIF. This is also true of *klf-4* and *c-myc* which both have been shown to contribute to the inhibition of ES cell differentiation [8, 9]. ES cells overexpressing Klf-4 demonstrated a trend toward reduced differentiation compared to wild-type cells but ultimately differentiated like their wild-type counterparts [9]. Similarly, ES cells overexpressing a hormone dependent Myc-ER, and propagated in the presence of tamoxifen, had the ability to self-renew in the absence of LIF but this property was progressively lost with time in culture [8]. Since none of these four genes is able to mimic the effect of LIF on ES cell self-renewal when it is individually overexpressed, it is likely that the four of them have to act cooperatively to block ES cell differentiation and maintain pluripotency. A synergistic action of Myc and Pim-1 would be particularly worthy of examination. The *c-myc* and *pim-1* genes are activated at comparable levels in response to LIF or to 4'OHT in the STAT3-ER^{T2} ES cells (data not shown). Myc is a transcriptional regulator of the *Cdc25A* gene [30] and Pim-1 phosphorylates, thereby activates, the phosphatase Cdc25A which is a positive G1-specific cell cycle regulator [24, 31]. Such a synergistic action of Myc and Pim-1 has been evidenced in lymphoid cells where it regulates Interleukin-6/STAT3-dependent proliferation [14].

Third, we showed that Pim-1 and Pim-3 regulate the growth rate of the population of undifferentiated ES cells. The method used is based on the calculation of the size of undifferentiated colonies obtained in self-renewal assays after knock-down of Pim-1 and

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Pim-3. This method allows to measure the growth rate of undifferentiated ES cells and, therefore, to distinguish the alterations in growth rates resulting from alterations in the proliferation rate of undifferentiated cells and those indirectly resulting from differentiation [19]. Inhibition of apoptosis is one mechanism by which Pim-1 and Pim-3 regulate the growth rate of undifferentiated ES cells. Pim kinases are known to play an important role in the prevention of cell death by inactivating the pro-apoptotic protein Bad [32, 33]. Anti-apoptotic effects of Pim kinases have been demonstrated in several experimental systems [14, 16]. LIF has been shown to inhibit apoptosis in ES cells [6, 34]. Activation of Pim-1 and Pim-3 expression could thus be one mechanism by which LIF exerts its anti-apoptotic effect.

To conclude, we provide evidence that the serine/threonine kinases Pim-1 and Pim-3 play an important in the maintenance of the ES cell identity. This findings contribute to further decipher the mechanisms by which the LIF/STAT3 pathway sustain self-renewal of mouse ES cells.

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Figure 1. (A) ES cells were induced to differentiate by withdrawal of LIF and formation of embryoid bodies for 1 to 6 days. Total RNA was subjected to RT-PCR analysis to evaluate expression of *pim-1*, *pim-2*, and *pim-3*. Expression of *Oct-4* was used as control of differentiation and *ß-actin* as control of the RT-PCR reaction. (B) Influence of LIF on *pim-1* and *pim-3* expression in CGR8 (left panel) and E14/T (right panel) ES cells. ES cells (+ LIF) were plated at a density of 2 x 10^6 cells per 10 cm dish, LIF-starved for 24 hrs (- LIF), then restimulated with 10,000 U/ml LIF for 1 hr. Histograms represent the levels of *pim-1* and *pim-3* mRNAs measured by real-time PCR in the "- LIF 24 hrs" and "+ LIF 1 hr" conditions normalized to the "+ LIF" condition [means and standard errors calculated from two (CGR8) or three (E14/T) replicates after normalization to *ß-actin*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, calculated with Student t-test]. (C) Immunoblots of total cell lysates revealed by anti-Cdc25A and anti-Pim-1 antibodies. Experimental conditions are described in (B). Numbers indicate levels of expression normalized to the "+ LIF" condition.
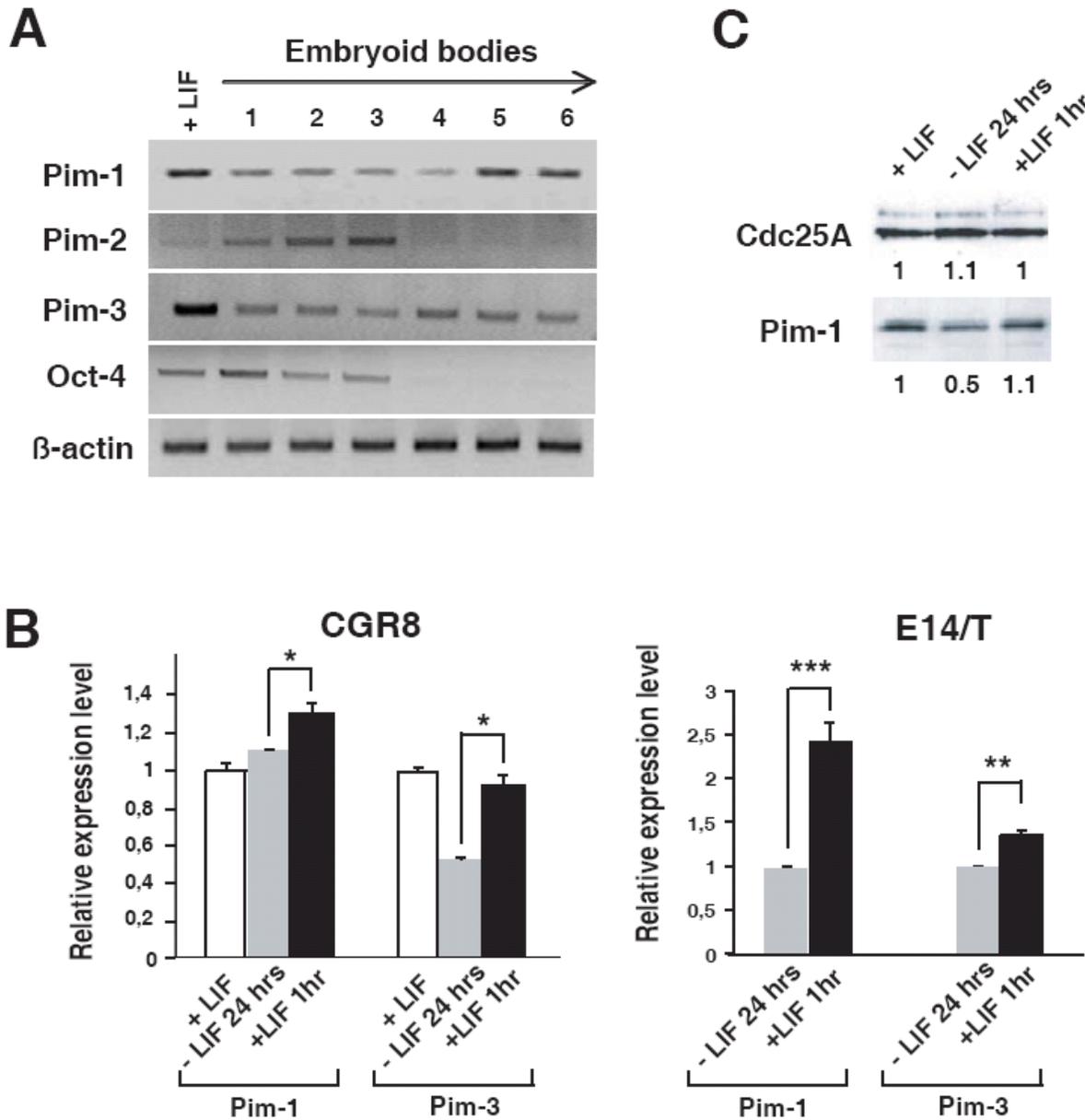
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Figure 2. Regulation of *pim-1* (A,B) and *pim-3* (C) expression in ES cells expressing GRgp278, GRgpY126-275F, and GRgpY118F chimaeric receptors. (A, B, C) ES cells (+ LIF) were deprived of LIF for 24 hrs (- LIF), then restimulated either with 10,000 U/ml LIF (+ LIF 1 hr) or with 30 ng/ml G-CSF (+ GCSF 1 hr). mRNA levels of *pim-1* and *pim-3* were analysed by real-time PCR and normalized with *ß-actin* cDNA. Histograms shown in (A) and (C) represent mRNA levels measured in the "- LIF", "+ LIF 1 hr" and "+ GCSF 1 hr" conditions and normalized to the "+ LIF" condition (means and standard errors calculated in three replicates; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, calculated with Student t-tests). (B) Immunoblots of total cell lysates revealed by anti-Pim-1 antibody. Numbers indicate levels of expression normalized to the "- LIF" condition.
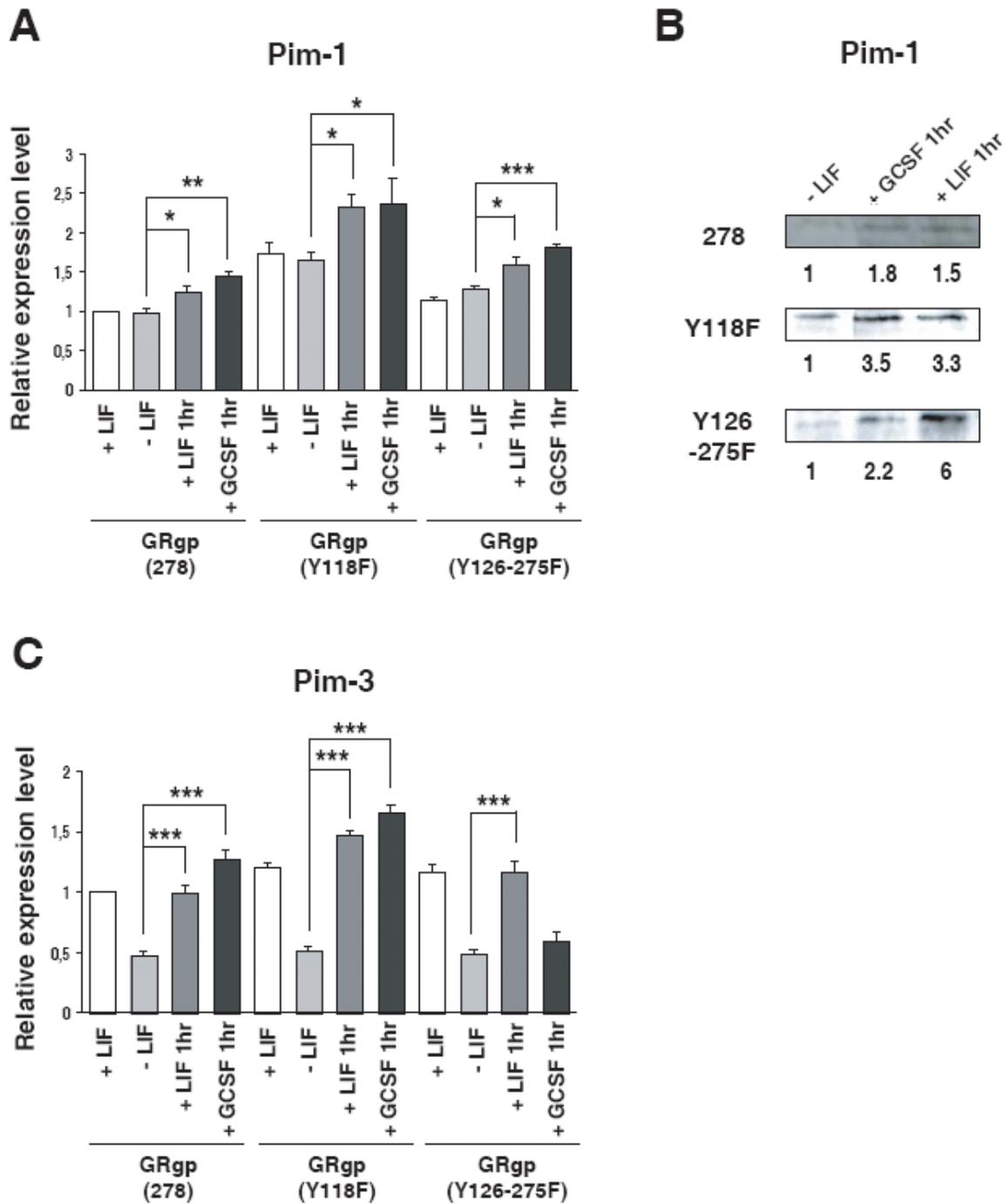
Figure 3. Regulation of *pim-1* and *pim-3* by hormone-dependent STAT3-ER^{T2}. E14/T cells expressing STAT3-ER^{T2} (left panel) or Cre-ER^{T2} (right panel) were deprived of 4'OHT for 24 hrs, then restimulated with 100 nM 4'OHT for 2 hrs in the presence of 100 µg/µl cycloheximide (CHX) or not. (A) mRNA levels of *pim-1* and *pim-3* were analysed by real-time PCR and normalized with *ß-actin* cDNA. Histogram represents mRNA levels measured in the "+ 4'OHT 2 hrs" and "+ 4'OHT 2 hrs + CHX" conditions and normalized to the "- 4'OHT" condition (means and standard errors calculated in three replicates; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, calculated with Student t-tests). (B) Pim-1 protein level analysed by immunoblotting with anti-Pim-1 antibody. Numbers indicate levels of expression in "+ 4'OHT 2 hrs" and "+ LIF 1hr" conditions normalized to the "- 4'OHT" condition.
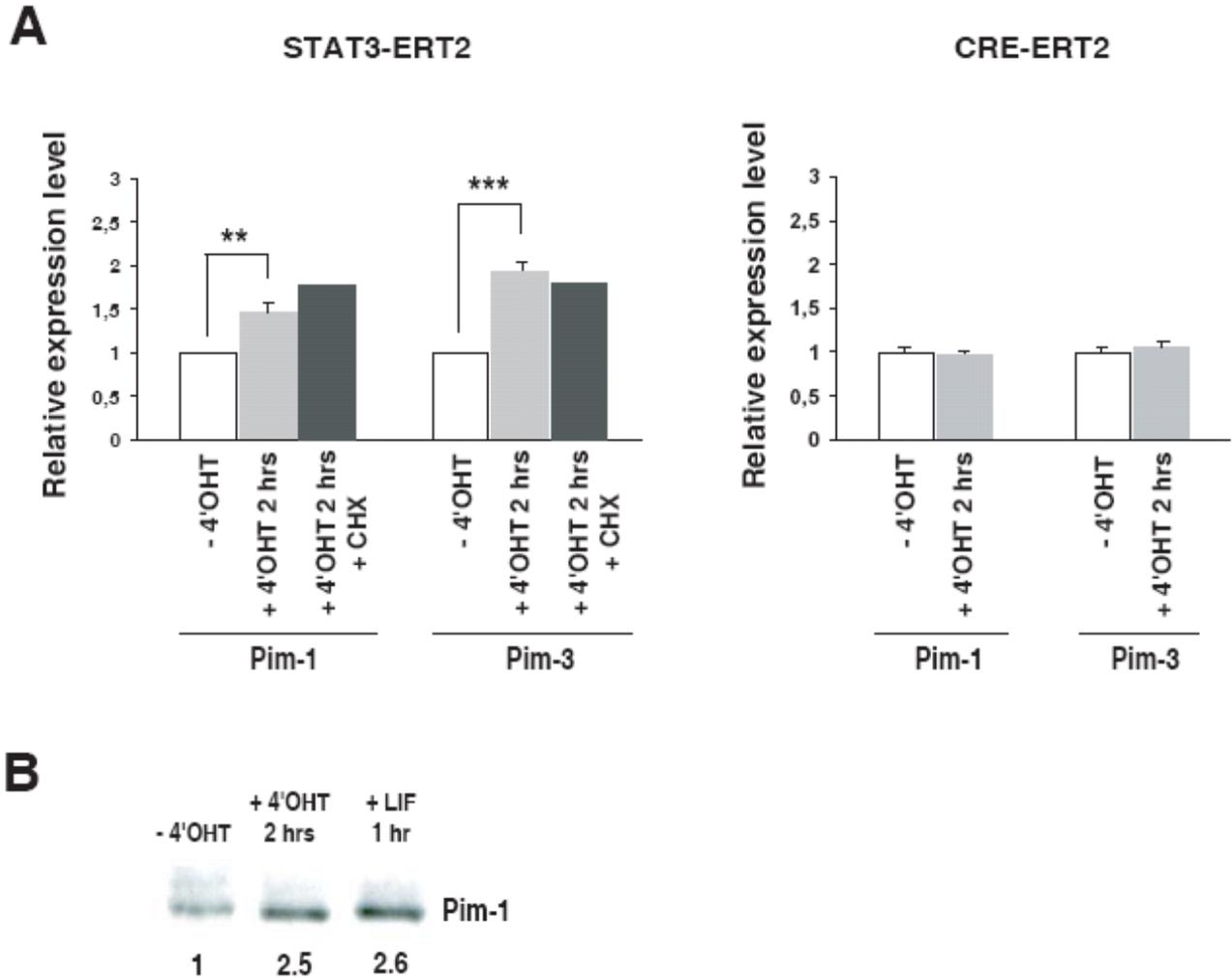
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Figure 4. Knock-down of *pim-1* and *pim-3* expression by lentiviral vector-mediated RNA interference and evaluation of self-renewal in a clonal assay. (A) Percentages of undifferentiated, mixed and differentiated colonies after staining to reveal AP activity. (B) Level of *Rex-1* mRNA in colonies harvested in (A), analysed by real-time PCR and normalized with *ß-actin* cDNA. Histogram represents *Rex-1* mRNA levels measured in colonies expressing shPim1, shPim3 and shSTAT3 after normalization to the *Rex-1* mRNA level measured in ES cells expressing shGFP. (C) *pim-1* and *pim-3* expression in ES cell clones resulting from infection with the lentiviral vectors expressing shPim-1 (top panel) and shPim-3 (bottom panel). Levels of *pim-1* and *pim-3* mRNAs in shPim-1 and shPim-3 clones are normalized to levels measured in a shGFP clone. (D) Percentages of undifferentiated, mixed and differentiated colonies after staining to reveal AP activity, obtained with shGFP clone 1, shPim-1 clones 1, 2 and 7, and shPim-3 clones 1, 3 and 5. (E) Level of *Oct4* and *Nanog* mRNAs in colonies harvested in (D), analysed by real-time PCR and normalized with *ß-actin* cDNA. Histogram represents *Oct4* and *Nanog* mRNA levels measured in colonies expressing shPim-1 (clones 1, 2 and 7), and shPim-3 (clones 1, 3 and 5), after normalization to the *Oct4* and *Nanog* mRNA levels measured in colonies expressing shGFP (clone 1). (F) Percentage of SSEA-1$^+$ cells in colonies harvested in (D) and analysed by flow cytometry. (A, D, F) Means and standard errors were calculated from three replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, calculated with Student t-tests (A, F) anf X^2-test (D).
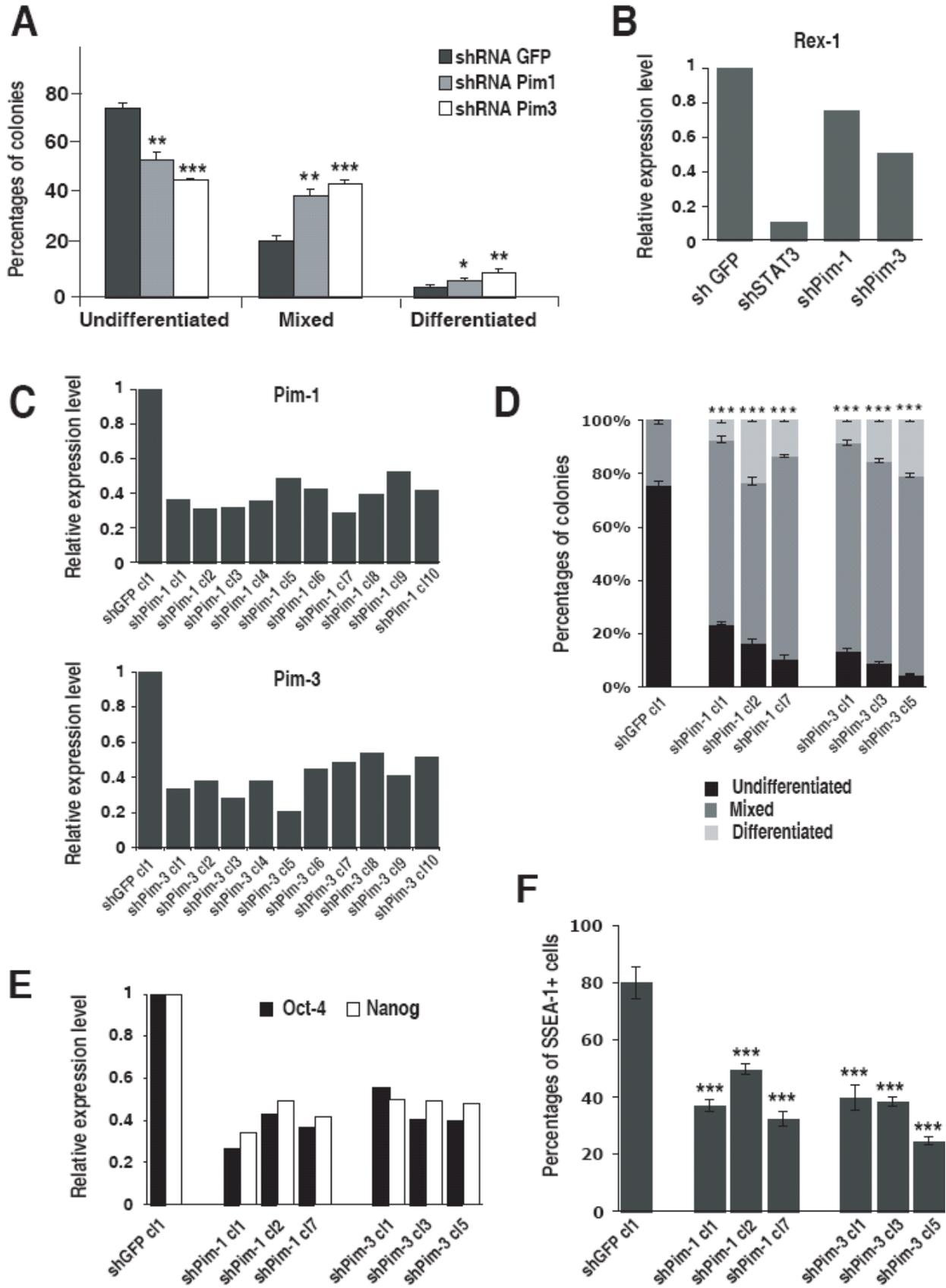
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Figure 5. Overexpression of Pim-1 and Pim-3 by means of episomal supertransfection. (A) E14/T ES cells were electroporated with *pPHPGK* (control), *pPHPGK-Pim1*, and *pPHPGK-Pim3* episomal vectors. ES cell transfectants were subsequently plated at 1 x 10^3 cells per 100 mm tissue culture dish and further cultured in complete ES cell medium for 7 days. Histograms represent the means and standard errors, measured in three replicates, of the percentages of undifferentiated, mixed and differentiated colonies. (B) E14/T ES cells were electroporated with *pPHCAG, pPHCAG-Pim1*, *pPCAGIZ*, and *pPCAGIZ-Pim3* episomal vectors, and propagated in culture medium supplemented with hygromycin B (*pPHCAG* and *pPHCAG-Pim1*) or with zeocine (*pPCAGIZ* and *pPCAGIZ-Pim3*) for 15 days. The four ES cell transfectants were subsequently plated at 1 x 10^3 cells per 100 mm tissue culture dish. Cells were exposed to medium without LIF for 0, 12, 24, 36 or 48 hrs and were subsequently cultured in complete ES cell medium with 1000 U/ml of LIF for 5 to 7 days ("LIF rescue" assay). Curves are based on the variations in the proportion of undifferentiated colonies as a function of the duration of LIF withdrawal in *pPHCAG* (control) and *pPHCAG-Pim1* transfectants (left panel), or in *pPCAGIZ* (control) and *pPCAGIZ-Pim3* transfectants (right panel) (thereafter named the four "conditions"). The proportion of undifferentiated colonies as a function of the duration of LIF removal was modelled using logistic regression (Generalized Linear Model with a binomial family). According to the this model, the proportions, p, transformed as *logit(p) = ln(p/(1 - p))* are related linearly to the explanatory variables, gene and time. However, we noted a systematic oscillation in the residuals as a function of time for each condition on the transformed scale due to the upper and lower asymptotes differing from 1 and 0, respectively. This deviation from linearity was well described by a fixed sine wave across the four conditions with frequency, f, estimated to be 0.025 cycles/hr (a period of about 40 hrs) over the four conditions. This value was used in the subsequent analysis. The final model required four estimates of intercept and slope (a, b), one for each condition, and one estimate of the sine and cosine coefficients (c, d) for the four conditions [*logit(p) = a + b * time + c * sin(2 * pi * f * time) + d * cos(2 * pi * f * time)*]. After taking account of differences in intercept and the residual oscillatory component, the differences in slopes between each group were significant, indicating that overexpression of Pim-1 and Pim-3 decreased the rate of decrease in the proportion of undifferentiated colonies as a function of the duration of LIF starvation (Pim-1 *vs* control: diff = 0.02, se = 0.003, z = 5.75, p < 0.001; Pim-3 *vs* control: diff = 0.009, se = 0.004, z = 2.08, p < 0.05). The figure shows the data on the logit scale after normalization by the intercepts and removal of the oscillatory component with the best fitting straight lines for each condition.
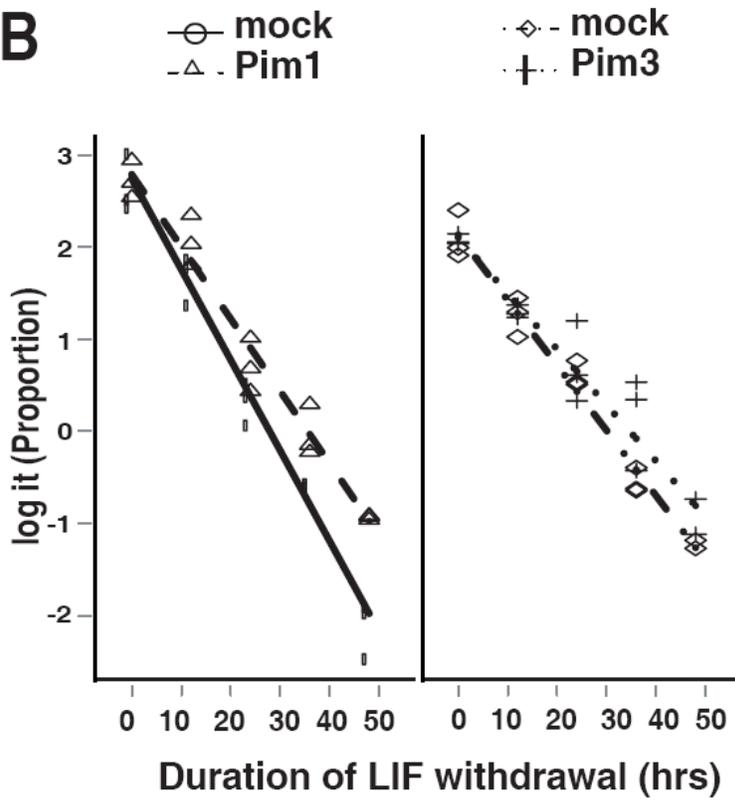
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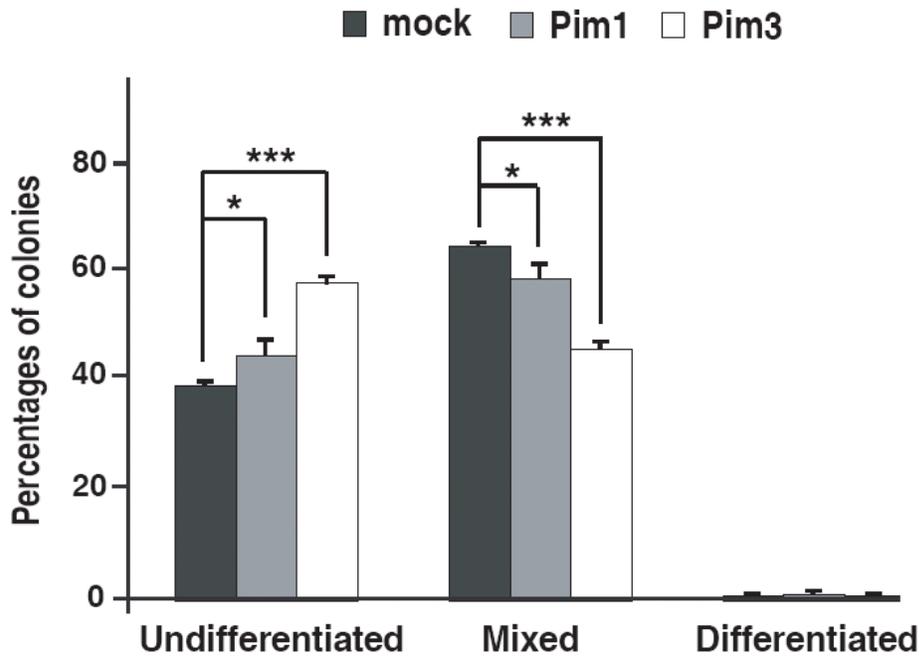
Figure 6. Knock-down of *pim-1* and *pim-3* expression by lentiviral vector-mediated RNA interference and evaluation of colony size (A) and apoptosis (B, C) in a clonal assay. The ES cells colonies expressing shPim-1, shPim-3 and shGFP were obtained as described in Figure 4A. (A, B) Histograms represent the means and standard errors, measured in three replicates, of the surface of undifferentiated AP^+ colonies (A) and the percentage of TUNEL-positive cells (B) (*, $p < 0.05$; ***, $p < 0.001$, calculated with Student t-tests). (C) Photomicrographies of undifferentiated colonies expressing shGFP, shPim-1 and shPim-3, showing TUNEL-positive cells stained with Hoechst (representative fields) (bar: 5 μM).
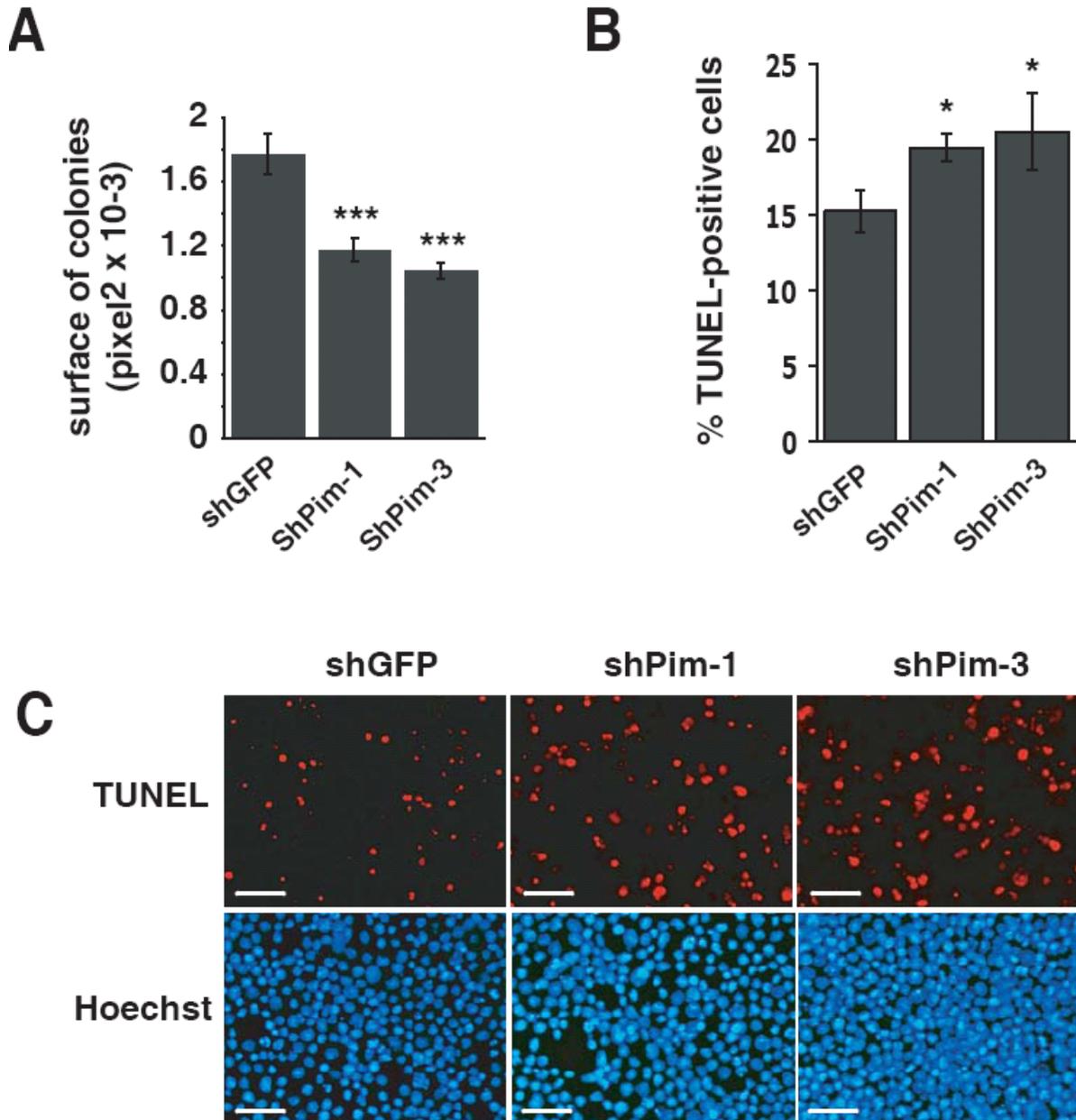
Self-renewal of murine embryonic stem (ES) cells is supported by the serine/threonine kinases Pim-1 and Pim-3

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Stem Cells published online Aug 23, 2007;
DOI: 10.1634/stemcells.2007-0066

This information is current as of August 28, 2007

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