FEBS Letters 584 (2010) 4241–4246

FEBS Letters

journal homepage: www.FEBSLetters.org

SHP2 is a downstream target of ZAP70 to regulate JAK1/STAT3 and ERK signaling pathways in mouse embryonic stem cells

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ARTICLE INFO

Article history:
Received 28 June 2010
Revised 8 September 2010
Accepted 8 September 2010
Available online 17 September 2010

Edited by Ned Mantei

Keywords:
ZAP70
SHP2
ERK1/2
LIF receptor
Self-renewal

ABSTRACT

Previous research indicated that ZAP70, a Syk family tyrosine kinase, is expressed in mouse embryonic stem cells (mESCs) and regulates the Janus kinase 1 (JAK1)/signal transducer and activator of transcription 3 (STAT3) signaling through consolidating SHP1 enzymatic activity. In this study, we report that SHP2 is another downstream target of ZAP70 in mESCs. We found that SHP2 phosphorylation and enzymatic activity are affected by Zap70 expression. In addition, we present evidence that ERK pathways activated by ZAP70 and SHP2 reduce the protein level of leukemia inhibitory factor (LIF) receptor. Based on these results, we propose that SHP2 is an essential mediator of the ZAP70 signal to regulate JAK1/STAT3 and ERK pathways in undifferentiated mESCs.

1. Introduction

Intracellular signal transduction pathways are essential for mouse embryonic stem cells (mESCs) grown in the presence of leukemia inhibitory factor (LIF) to maintain an undifferentiated, self-renewing state of the cells [1]. LIF promotes the proliferation of undifferentiated stem cells through the activation of a heteromeric complex containing two related cytokine receptor subunits, gp130 and LIF receptor (LIFR) [2,3]. One of the most important pathways activated by LIF in mESCs is the Janus kinase 1 (JAK1)/signal transducer and activator of transcription 3 (STAT3) pathway [4,5]. Phosphorylation and dephosphorylation of JAK1 and STAT3 plays a central role in controlling the activity of the pathway. The majority of phosphorylation and dephosphorylation occurs at tyrosine residues and is carried out by protein tyrosine kinases and protein tyrosine phosphatases, respectively [4,6]. The Ras-Raf-mitogen activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) signaling cascade is another important pathway in ESCs. Stimulation of ERK1 and ERK2 is known to be of particular importance in inducing differentiation of mESCs [7,8]. The pluripotent state of ESCs is maintained by the balance between self renewal regulated by JAK1/STAT3 and differentiation potential regulated by the ERK pathway [7]. Therefore, a functional analysis of signals transduced through the JAK1/STAT3 and ERK pathways provides an opportunity to elucidate the molecular mechanisms that regulate stemness of ESCs.

Src homology region 2 domain-containing phosphatase 2 (SHP2) is a cytoplasmic phospho-tyrosine phosphatase with two Src-homology 2 (SH2) domains at the N-terminus that modulates signal strength downstream of cytokine/growth factor receptors [9,10]. In mESCs, SHP2 appears to negatively regulate the activity of STAT3. *Shp2*Δ/Δ mutant mESCs showed defective differentiation and more efficient self-renewal in the presence of LIF, which was at least in part due to increased STAT3 activity in the absence of functional SHP2 [11,12]. In contrast to the phospho-STAT3 signal, the LIF-stimulated phospho-ERKs level is decreased in SHP2-deficient mESCs compared to wild-type cells [11,12], which suggests that SHP2 has a reciprocal role in modulating LIF-stimulated ERK and STAT3 pathways in mESCs. Furthermore, the function of SHP2 to regulate pluripotency and differentiation of mESCs is conserved with hESCs, despite that LIF is not required for hESC self-renewal [13].

ZAP70, a Syk family tyrosine kinase, has been reported to be present exclusively in normal T cells and natural killer (NK) cells, serving as a pivotal regulator of antigen-mediated receptor signaling [14]. A recent study suggested that Zap70 is expressed in mESCs and functions as a key upstream regulator of JAK1/STAT3 signaling in the cells [15]. In particular, ZAP70 modulates SHP1

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0014-5793/$36.00
doi:10.1016/j.febslet.2010.09.016

enzymatic activity to inhibit excess JAK1/STAT3 signaling in LIF-stimulated culture conditions [15]. In this study, for the first time to our knowledge, we report that ZAP70 regulates SHP2 activity in undifferentiated mESCs. We demonstrate that SHP2 is a mediator of the ZAP70 signal to regulate JAK1/STAT3 and ERK pathways in mESCs. Furthermore, ERKs activity regulated by ZAP70 and SHP2 signal modulates LIFR protein level in mESCs.

2. Materials and methods

2.1. Cell culture and transfection

J1 mESCs (cat # SCRC-1010) were purchased from ATCC (www.atcc.org). Zap70 knocked down mESC (Zap70KD) were generated as described previously [15]. mESCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal calf serum (Hyclone), 0.1 mM 2-mercaptoethanol (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Gibco) and 1000 U/ml LIF (Chemicon). For transient knockdown of Zap70 and Shp2, ON-TARGET plus siRNA mixture (Dharmacon) for each gene was transfected into mESCs as described previously [15].

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted using TRIzol (Invitrogen), and 2–5 μg of total RNA was reverse-transcribed using the SuperScriptII™ First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions.

2.3. Protein extraction and immunoblot

Immunoblotting was performed as previously described [16]. The following antibodies were used: STAT3 (sc-482, Santa Cruz), phospho-STAT3 (Tyr-705) (#9131, Cell Signaling), ERKs (sc-154, Santa Cruz), phospho-ERKs (Thr 202/Tyr 204, Sigma), SHP2 (sc-280, Santa Cruz), phospho-SHP2 (#3703, Cell Signaling), c-MYC (sc-764, Santa Cruz), LIFR (sc-659, Santa Cruz), and β-actin (sc-47778, Santa Cruz). Immunoreactivity was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, England). For quantitative analysis, the mean density of each band was measured with Multi Gauge V3.0 software, and the band density of phospho-SHP2 and phospho-ERKs was divided by the density of the corresponding total protein band to obtain the normalized band density. The immunoblot band density of c-MYC and LIFR was divided by that of β-actin.

2.4. Immunoprecipitation and phosphatase assay

Cell lysates containing 1 mg of protein were diluted to 500 μl with lysis buffer and incubated with specific antibodies against SHP2 or ZAP70 (Santa Cruz) overnight at 4 °C. Immunocomplexes were recovered by incubation with 20 μl of a 50% slurry of protein-A-conjugated Sepharose (Amersham Biosciences, Inc.) in PBS for 4 h with agitation. Phosphatase activity was determined colorimetrically using *p*-nitrophenylphosphate (*p*NPP; Sigma) as a substrate. The rate of hydrolysis of *p*NPP was analyzed by measuring the change in absorbance at 410 nm. Beads were washed twice with cold PBS and boiled in SDS sample buffer, separated by SDS–PAGE, and immunoblotted to detect the presence of SHP2.

2.5. Statistical analysis

Graphical data are presented as the means ± standard deviations (S.Ds). Statistical significance was determined using Student's *t*-test. Statistical analyses were performed using the SAS statistical package v.9.13 (SAS Inc., Cary, NC, USA, http://www.sas.com/).

3. Results

3.1. The effect of Zap70 suppression on the phosphorylation and enzymatic activity of SHP2 in mESCs

Zap70 is expressed in mESCs and functions to maintain stemness of mESCs by negatively regulating JAK1/STAT3 signaling [15]. Because SHP2 is one of the known negative regulators of Jak/STAT3 signaling [7], we hypothesized that SHP2 is a downstream target of ZAP70 in mESCs. To address this possibility, we first tested whether a chemical inhibitor of ZAP70 activity blocked phosphorylation of Tyr580 in the C-terminal tail of SHP2, which is positively related to the enzymatic activity of SHP2 [17]. As shown in Fig. 1A, piceatannol, a potent Syk/ZAP70-specific tyrosine kinase inhibitor [18], abrogated SHP2 Tyr580 phosphorylation gradually in a concentration-dependent manner. Consistent with this finding, a significantly lower level of phospho-SHP2 was observed in Zap70 knocked down mESC cell line (Zap70KD) (Fig. 1B). SHP2 phosphorylation was also suppressed by the transient knockdown of Zap70 (Fig. 1C). Based on the fact that SHP2 Tyr580 phosphorylation is directly related to the enzymatic activity of SHP2 [18], we speculated that Zap70KD may exhibit altered SHP2 phosphatase activity. In support of this idea, the enzymatic activity of SHP2, immunoprecipitated from Zap70KD lysates, was significantly reduced compared to that of the control (Fig. 1D).

3.2. The effect of Zap70 overexpression on the phosphorylation and enzymatic activity of SHP2 in mESCs

The effects of Zap70 knockdown on the phosphorylation and phosphatase activity of SHP2 suggest that the enzymatic activity of SHP2 is regulated by the kinase activity of ZAP70 in mESCs. To further substantiate this possibility, we over-expressed Zap70 transiently in mESCs (OE), and then analyzed phosphorylation and enzymatic activity of SHP2. As expected, Zap70 overexpression induced SHP2 Tyr580 phosphorylation (Fig. 2A). We found that SHP2 activity in Zap70OE was significantly increased compared to the control cells (Fig. 2B). As reported previously, Zap70OE shows reduced levels of STAT3 phosphorylation and c-MYC expression (Fig. 2C, left panel) [15]. When SHP2 expression was suppressed by siRNA, the effect of ZAP70 toward both STAT3 phosphorylation and c-MYC expression was reverted (Fig. 2C, right panel). Taken together, these results support the idea that SHP2 activity is affected by ZAP70 in mESCs, which subsequently leads to regulation of the JAK1/STAT3 signaling pathway. Previously, we reported that enzymatic activity of SHP1 is affected by ZAP70 in mESCs [15]. Therefore, our results suggest that the negative effect of ZAP70 on the JAK1/STAT3 pathway in mESCs is caused by SHP2 in conjunction with SHP1 activity.

3.3. ZAP70 represses LIFR expression through the SHP2 and ERK signaling pathways in mESCs

Zap 70 knockdown induces increased self-renewal of mESCs by enhancing STAT3 signaling [15]. Because LIF-stimulated ERK and STAT3 signals are finely tuned in opposite directions to maintain the stemness of mESCs, we hypothesized that ZAP70 may activate the ERK pathway in mESCs. To address this, we examined ERK phosphorylation in mESCs following chemical inhibition of ZAP70 activity. As expected, the level of phospho-ERKs was decreased as piceatannol concentration increased (Fig. 3A). On the contrary, phospho-ERKs were increased upon Zap70 overexpression in

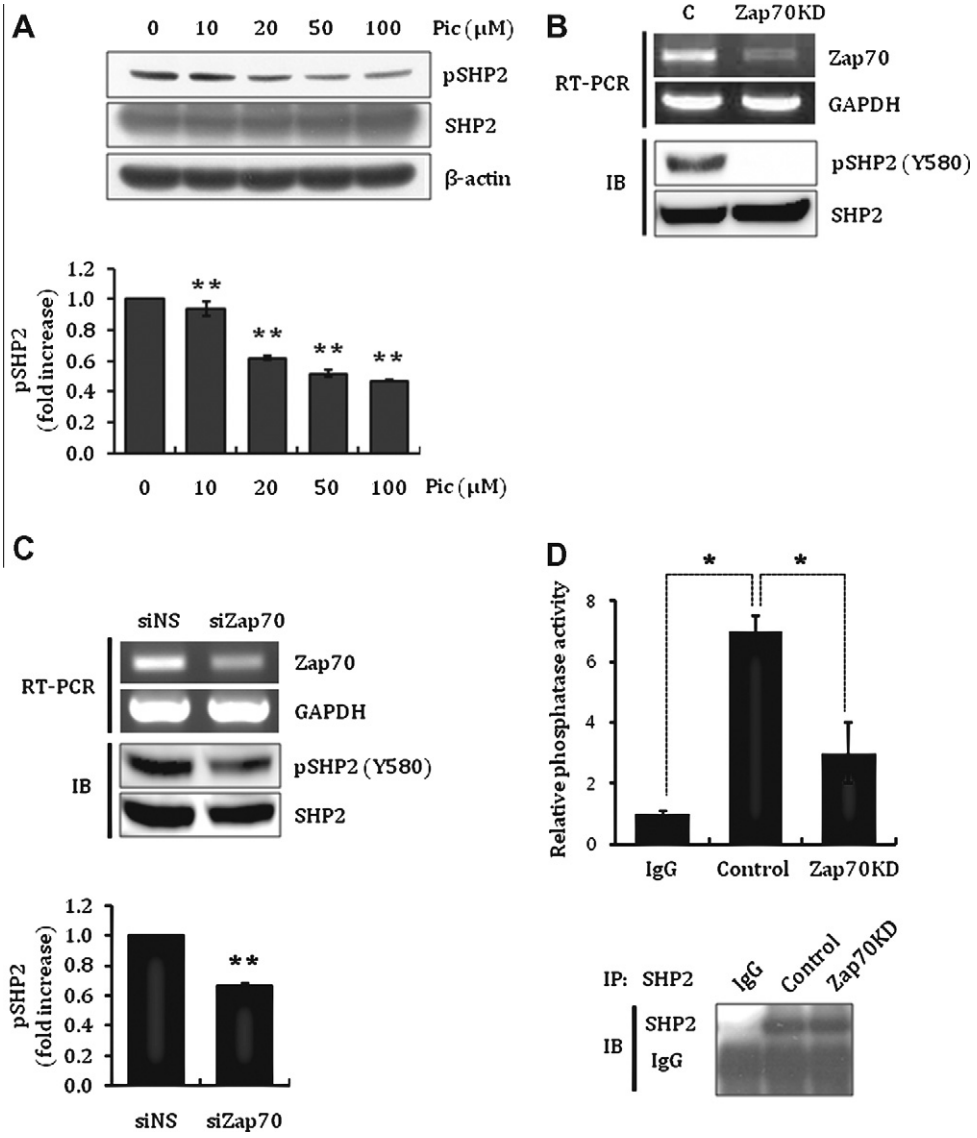

Fig. 1. The effect of Zap70 suppression on the phosphorylation and enzymatic activity of SHP2. (A) Piceatannol was added to the culture media of mESCs for 24 h at the indicated concentrations. Total and phosphorylated SHP2 were analyzed by immunoblot. (B) Phosphorylation of SHP2 in Zap70 knockdown mESC cell line [15] was analyzed by immunoblot. (C) Transient knockdown of Zap70 was induced by the transfection of siRNA targeting Zap70, and the phosphorylation of SHP2 was then analyzed by immunoblot. (D) Total protein from control or Zap70 knocked down mESCs was immunoprecipitated by anti-SHP2 antibody and phosphatase activity was analyzed (top panel). SHP2 protein level in immunoprecipitated samples was determined by immunoblot (bottom panel). * Indicates significant ($P < 0.05$) and ** indicates highly significant ($P < 0.01$) results based on Student's *t*-test. Error bars represent standard deviation from at least three independent experiments. In (B) and (C), Zap70 expression level was analyzed by RT-PCR. *Abbreviations*: Pic, piceatannol; C, stable cell line of mESCs constructed by the genomic integration of non-specific shRNA plasmid; Zap70KD, stable cell line of Zap70 knocked down mESCs; siZap70, siRNA targeting Zap70; siNS, non-specific siRNA; IP, immunoprecipitation; IB, immunoblot.

mESCs (Fig. 3B). When siRNA targeting Shp2 was cotransfected with Zap70 expressing plasmid, the effect of ZAP70 on the phosphorylation of ERKs was abolished (Fig. 3C). This result implies that the enhanced phosphorylation of ERKs in Zap70OE is caused by SHP2 activity. Previously, we reported that the expression of LIFR is significantly upregulated in Zap70KD [15]. In neuroblastoma cells, stimulation with epidermal growth factor or fibroblast growth factor decreases LIFR in an ERK1/2-dependent manner and the downregulation is due to increased rates of lysosomal degradation [19]. Likewise, the inhibition of ERK2 activity with U0126 induced an increase of LIFR protein in mESCs (Fig. 3D). Given that phosphorylation of ERKs is regulated by the ZAP70 and SHP2 signaling pathway, we examined whether the ZAP70 effect on LIFR regulation is caused by SHP2 activity. As expected, Zap70 overexpression caused suppression of LIFR (Fig. 3E). However, when Shp2 was knocked down by siRNA, the suppression effect of ZAP70 on the LIFR level was reversed (Fig. 3F). Taken together, these results suggest that ZAP70 represses LIFR expression through the activation of SHP2.

4. Discussion

SHP2 functions physiologically as a molecular switch governing self-renewal versus differentiation in mESCs. It modulates LIF-stimulated STAT3 and ERK signals bi-directionally [20]. In this study, we suggest that ZAP70 is an upstream regulator of SHP2 phosphatase, which suppresses STAT3 phosphorylation and enhances ERK1/2 phosphorylation (Fig. 4). Furthermore, we suggest that the ZAP70 and SHP2 pathway modulates LIFR expression via the ERK1/2 pathway. Our Zap70OE and siShp2 analysis shows that SHP2 and ERK1/2 activity is up-regulated by ZAP70, resulting in the suppression of LIFR expression in mESCs. In neuroblastoma

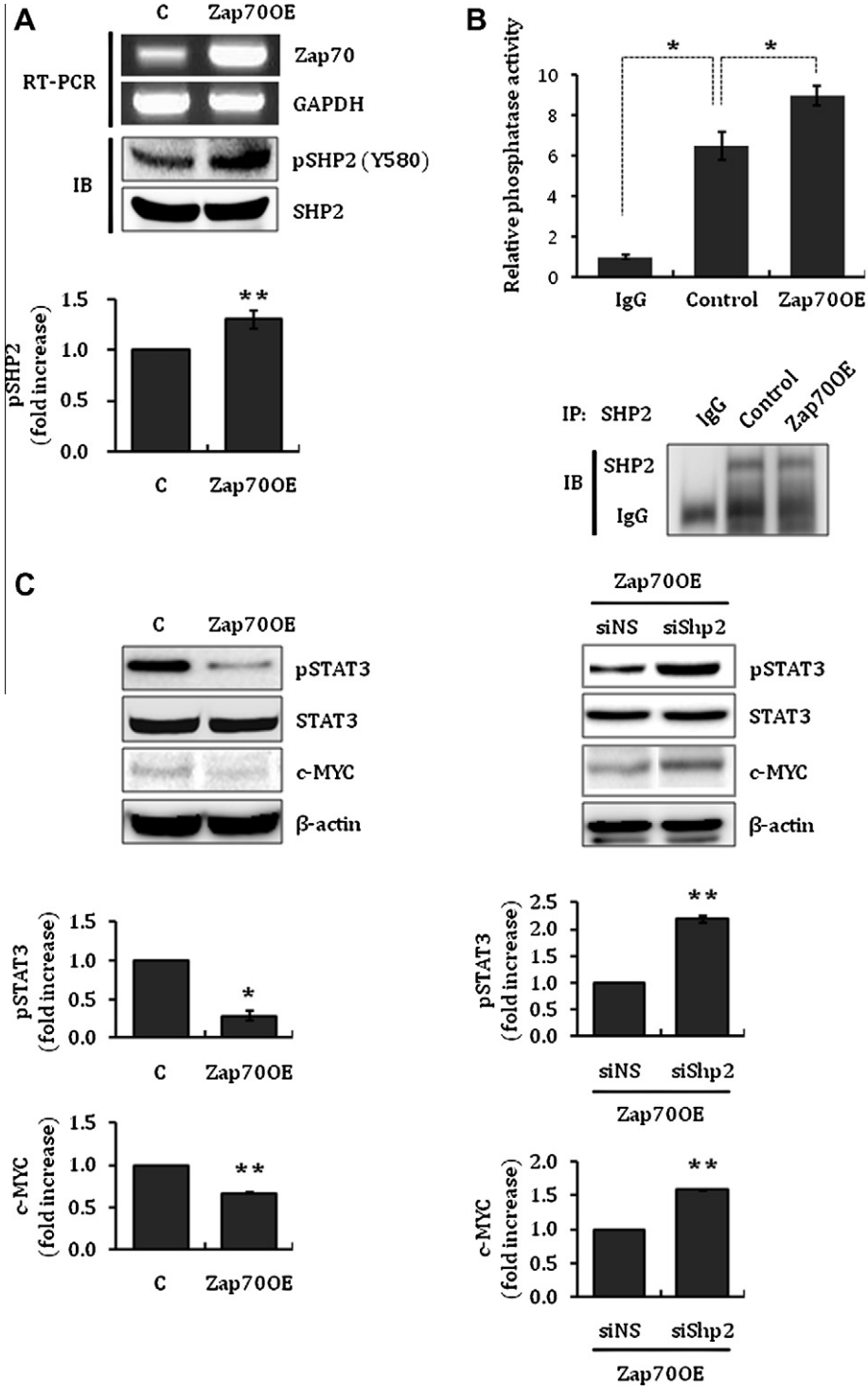

Fig. 2. The effect of Zap70 overexpression on the phosphorylation and enzymatic activity of SHP2. (A) Zap70-expressing plasmid was transiently transfected into mESCs for 48 h, Zap70 expression was analyzed by RT-PCR and phosphorylation of SHP2 was analyzed by immunoblot. (B) Total protein of mESCs transfected with GFP or Zap70 expressing plasmid was immunoprecipitated by anti-SHP2 antibody and phosphatase activity was analyzed (right panel). SHP2 protein level in immunoprecipitated samples was determined by immunoblot (left panel). (C) Total proteins of mESCs transfected with GFP or Zap70 expressing plasmid were analyzed for the phosphorylation of STAT3 or c-MYC expression (left panel). Zap70 expressing plasmid was cotransfected with siNS or siShp2 into mESCs for 48 h. Phospho-STAT3, STAT3 and c-MYC expression were then analyzed by immunoblot. * Indicates significant ($P < 0.05$) and ** indicates highly significant ($P < 0.01$) results based on Student's *t*-test. Error bars represent standard deviation from at least three independent experiments. *Abbreviations*: C, mESCs transfected with GFP expressing plasmid; Zap70OE, mESCs transfected with Zap70 expressing plasmid; siShp2, siRNA targeting Shp2; siNS, non-specific siRNA; IP, immunoprecipitation; IB, immunoblot.

cells, the level of LIFR is regulated by lysosomal degradation depending on ERK1/2 activity as a negative mechanism [19]. Therefore, further investigation is required to demonstrate the underlying mechanism of ZAP70 to regulate LIFR level in mESCs.

Recently, it was reported that the function of SHP2 to regulate differentiation of ES cells is conserved between mESCs and hESCs, despite that the molecular mechanism to sustain self-renewal of the two types of ES cells is different [13]. Since our preliminary

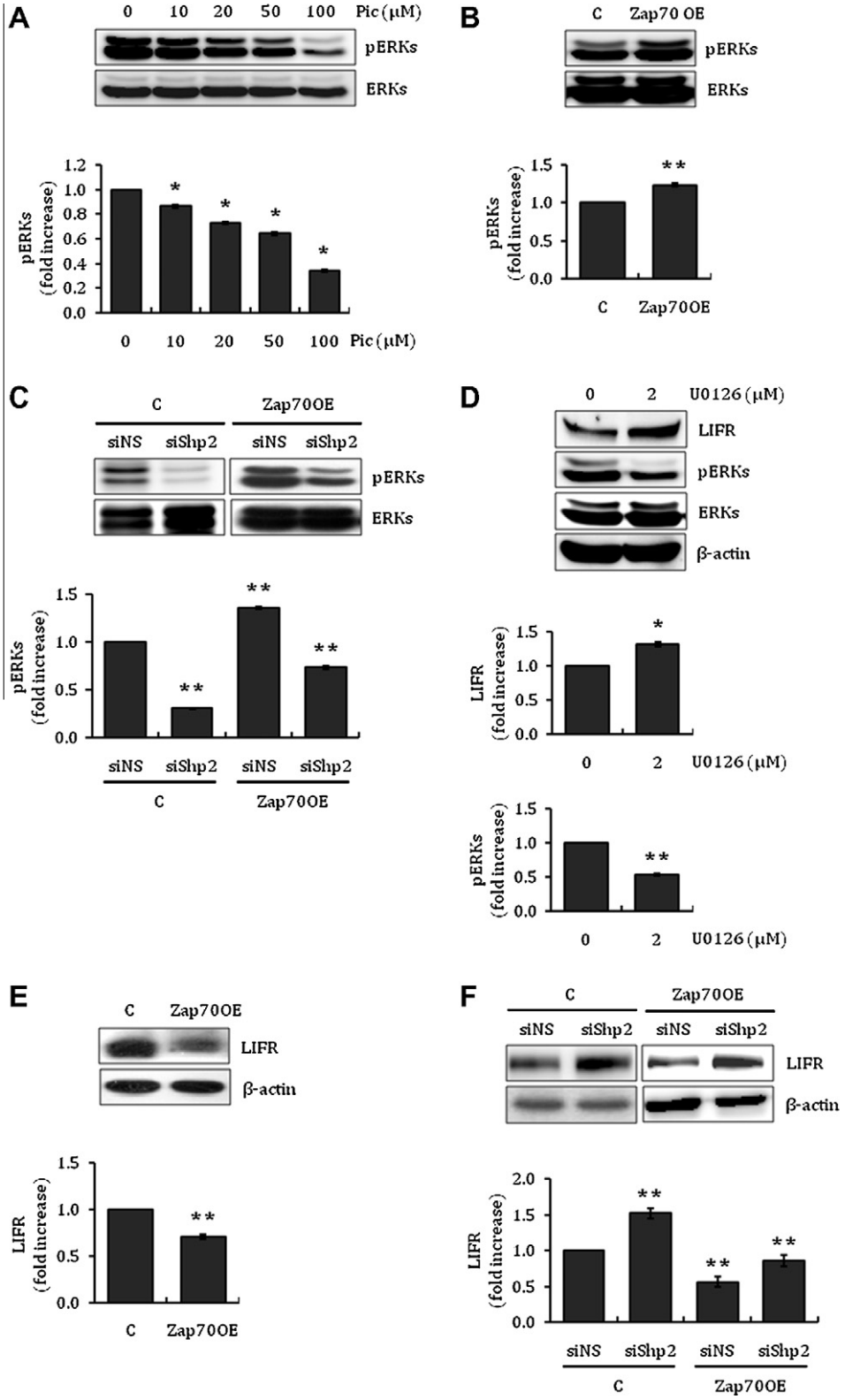

Fig. 3. ZAP70 represses LIFR expression through SHP2 in mESCs. (A) Piceatannol was added to the culture media of mESCs for 24 h at the indicated concentrations and phospho-ERK1/2 level was analyzed by immunoblot. (B) Total proteins of mESCs transfected with GFP or Zap70 expressing plasmid were analyzed for ERK1/2 phosphorylation. (C) GFP or Zap70 expressing plasmid was cotransfected with siNS or siShp2 into mESCs for 48 h. phospho-ERK1/2 level was analyzed by immunoblot. (D) 0 or 2 μM of U0126 was added to the culture media of wild-type mESCs for 24 h and LIFR expression was analyzed by immunoblot. (E) GFP or Zap70 expressing plasmid was transfected into mESCs for 48 h and LIFR level was analyzed by immunoblot. (F) GFP or Zap70 expressing plasmid was cotransfected with siNS or siShp2 into mESCs for 48 h and LIFR level was analyzed by immunoblot. * Indicates significant ($P < 0.05$) and ** indicates highly significant ($P < 0.01$) results based on Student's *t*-test analyses. Error bars represent standard deviation from at least three independent experiments. *Abbreviations*: Pic, piceatannol; C, mESCs transfected with GFP expressing plasmid; Zap70OE, mESCs transfected with Zap70 expressing plasmid; siShp2, siRNA targeting Shp2; siNS, non-specific siRNA.

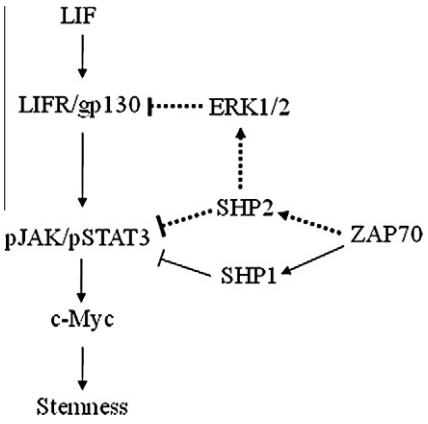

Fig. 4. Proposed model of ZAP70 and SHP2 activity in mESCs. ZAP70 phosphorylates Tyr580 of SHP2 to activate phosphatase activity of SHP2, which subsequently leads to the dephosphorylation of STAT3, as well as activation of the ERK pathway and consequent suppression of LIFR protein level. The binary activity of SHP2 thus influences "stemness" in mESCs.

experiment revealed that Zap70 is expressed in undifferentiated hESCs (data not shown), it will be of great interest to elucidate whether ZAP70 is an upstream regulator of SHP2 in hESCs to regulate the switch between pluripotency and differentiation of the cells.

Phosphorylation of ERK1/2 is enhanced upon Zap70 overexpression, which suggests that ERK1/2 is not the direct target of SHP2. Because Sprouty, which is a conserved inhibitor of the Ras-MAP kinase signaling pathway, is a known target of SHP2 [21], it will be important to elucidate whether SHP2 functions as a positive regulator of ERK1/2 pathway by inactivating Sprouty protein in mESCs.

Acknowledgements

This work was supported by the Korea Science and Engineering Foundation (KOSEF) Grant funded by the Korean government (MOST) (2009-0083688 and 20100016803).

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