Ell3 Enhances Differentiation of Mouse Embryonic Stem Cells by Regulating Epithelial-Mesenchymal Transition and Apoptosis

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Abstract

Ell3 is a testis-specific RNA polymerase II elongation factor whose cellular function is not clear. The present study shows that Ell3 is activated during the differentiation of mouse embryonic stem cells (mESCs). Furthermore, Ell3 plays a critical role in stimulating lineage differentiation of mESCs by promoting epithelial-mesenchymal transition (EMT) and suppressing apoptosis. Mouse ESCs engineered to stably express Ell3 were rapidly differentiated compared with control cells either under spontaneous differentiation or neural lineage-specific differentiation conditions. Gene expression profile and quantitative RT-PCR analysis showed that the expression of EMT markers, such as Zeb1 and Zeb2, two major genes that regulate EMT, was upregulated in Ell3-overexpressing mESCs. Remarkably, knockdown of Zeb1 attenuated the enhanced differentiation capacity of Ell3-overexpressing mESCs, which indicates that Ell3 plays a role in the induction of mESC differentiation by inducing EMT. In contrast to Ell3-overexpressing mESCs, Ell3-knock out mESCs could not differentiate under differentiation conditions and, instead, underwent caspase-dependent apoptosis. In addition, apoptosis of differentiating Ell3-knock out mESCs was associated with enhanced expression of p53. The present results suggest that Ell3 promotes the differentiation of mESCs by activating the expression of EMT-related genes and by suppressing p53 expression.

Introduction

Pluripotency refers to the capacity of embryonic stem cells (ESCs) to differentiate into all cell types [1,2]. ESCs possess self-renewal capacity, which is the ability to proliferate for prolonged periods while maintaining the undifferentiated state. Recently, a core set of transcription factors, including Oct4, Sox2, and Nanog were found to upregulate the expression of genes that control self-renewal while repressing genes that drive differentiation [3–6]. How ESCs overcome the constraints of their self-renewal machinery and initiate differentiation is of great interest because understanding the mechanisms underlying differentiation will facilitate the therapeutic application of ESCs in promoting lineage-specific differentiation. The findings of recent studies have led to major advances in the molecular and biochemical understanding of the transition of ESCs from the self-renewal state to early differentiation. A recent report showed that the transcriptional repressor Rost, which is abundantly expressed in ESCs and is a target of the Oct3/4-Sox2-Nanog regulatory network, is not required for the maintenance of ES cell pluripotency, but promotes cell differentiation by suppressing self-renewal genes [7].

Several signaling networks including the leukemia inhibitory factor (LIF)/Stat3, Bmp/Smad, Ras/MAPK and Calcineurin-NFAT pathways also regulate the molecular switch between ESC self-renewal and differentiation [8–12]. For example, Zap70 functions to modulate the balance between LIF/Stat3 and Ras/MAPK pathways to maintain the pluripotent differentiation capacity of mouse ESCs (mESCs) [13,14].

In addition to transcription factors and signaling pathways, epigenetic processes such as DNA methylation and chromatin remodeling are essential for determining cell fate between self-renewal and differentiation [15]. However, while recent studies on the mechanisms underlying the maintenance of the self-renewing pluripotent state have improved our understanding of ESCs, how ESCs initially enter into lineage commitment is still only partially understood.

Epithelial cells form coherent tissue layers because their lateral membranes are closely attached by intercellular adhesion complexes such as tight junctions, adherens junctions, and gap junctions, whereas mesenchymal cells can move as individual cells throughout the extracellular matrix because they are nonpolarized and lack intercellular junctions [16]. Epithelial-mesenchymal transition (EMT) is the phenotypic transformation of epithelial cells into mesenchymal cells and is related to various biological changes in development and disease. Recently, it was described that calcineurin-NFAT signaling promotes EMT during the switch of ESCs from an undifferentiated state to lineage differentiation [9]. Furthermore, several ESC-specific transcription factors were
shown to bind promoters of EMT-related genes [17]. Therefore, EMT appears to be an early and essential step in lineage specification of ESCs.

Ell is a 621-amino acid protein that functions as a transcription elongation factor by suppressing the transient pausing of RNA polymerase II at multiple sites on DNA from both promoter-dependent and promoter-independent templates [18]. Ell is a testis-specific RNA polymerase II elongation factor, which increases the catalytic rate of transcription elongation [19]. The C-terminal domain of Ell shares strong similarities to that of Ell, which acts as a negative regulator of p53 and regulates cell proliferation and survival [20,21].

Here, we analyzed the role of Ell in the differentiation of mESCs. We show that Ell-overexpressing mESCs rapidly differentiated compared with control cells. Furthermore, Ell-knock-down mESCs underwent apoptosis under differentiation conditions. We also demonstrate that Ell activates EMT-inducing genes, including Zeb1, and regulates the expression level of p53. Collectively, our results identify a unique function for Ell during the initiation of mESC differentiation, and we suggest that Ell promotes the differentiation of mESCs by inducing EMT and suppressing p53.

Materials and Methods

Reagents and cell culture

The mESC line J1 (cat. # SCRC-101) was purchased from ATCC (Manassas, VA, http://www.atcc.org). mESCs were maintained on 0.1% gelatin-coated dishes in Dulbecco’s modified Eagle’s medium (DMEM, Gibco Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 10% horse serum (Gibco Invitrogen), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco Invitrogen), 1× non-essential amino acids (Life Technologies), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St Louis, MI, http://www.sigmaaldrich.com), and 1,000 U/mL LIF (Chemicon, Temecula, CA, http://www.chemicon.com). To form embryonic bodies (EBs), mESC colonies were trypsinized to achieve a single-cell suspension and subsequently cultured on uncoated Petri dishes in ESC medium without LIF. To induce spontaneous differentiation, mESCs were cultured in LIF-deficient ESG medium (as described above) with 500 nM all-trans retinoic acid (RA).

Genetic modification of mESCs

Ell3-overexpressing (OE) mES cells lines were generated by chromosomal integration of an Ell3 expression plasmid, which was constructed by cloning PCR-amplified Ell3 cDNA into modified pCDNA3.1 vectors (Invitrogen, Carlsbad, CA) in which the CMV promoter was replaced with an EF1α promoter. ShRNA plasmids targeting mouse Ell3 were purchased (RMM3981-9849969, Open Biosystems, Huntsville, AL) and used to generate a stable targeting mouse Ell3 OE and Ell3 KD mESCs, respectively, and all experiments were repeated in each cell line to confirm the results. Non-specific control siRNAs were purchased from Bioneer (Daejeon, Korea), and siRNAs targeting Ell3 were purchased from Dharmacon (Denver, CO). mESCs were transfected with either siRNA or plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Neural differentiation of mESCs

For monoculture neural differentiation, undifferentiated ESCs were dissociated and plated onto 0.1% gelatin-coated tissue culture plates in ESC media. After 24 h, media was exchanged with neural differentiation medium prepared as a 1:1 mixture of DMEM/F12 (Gibco) supplemented with modified N2 (25 μg/mL insulin, 100 μg/mL apotransferrin, 6 ng/mL progesterone, 16 μg/mL putrescine, 30 nM sodium selenite, and 50 μg/mL bovine serum albumin fraction V) (Gibco) and neurobasal medium supplemented with B27 (both from Gibco). Medium was replaced every 2 days.

RNA extraction and real-time RT-PCR

Total RNA was prepared from mESCs using TRIzol (Invitrogen) and 2–5 μg of total RNA was reverse-transcribed into cDNA using the SuperScriptII™ First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was performed in triplicate with the Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA, http://www.qiagen.com) and CFX96 Real-time System (Bio-Rad Laboratories, Richmond, CA http://www.bio-rad.com). For quantification, target gene expression was normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. The PCR primers used in this study are listed in Table S1.

Immunoblotting

For protein analysis, cells were washed twice with cold phosphate buffered saline (PBS) and lysed with tissue lysis buffer (20 mM Tris-base, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride, and 1 mM benzamidine). Lysates were centrifuged at 20,000×g for 10 min to remove cellular debris. Whole-cell extracts were prepared and 50 μg of protein were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA; http://www.millipore.com) for detection with anti-p53 (#2524, Cell Signaling, Denver, MA; http://www.cellsignal.com), Caspase-3 (#9656, Cell Signaling), Caspase-9 (#9504, Cell Signaling), c-Myc (sc-764, Santa Cruz), Oct4 (sc-5279, Santa Cruz), Sox2 (sc-20088, Santa Cruz), Nanog (sc-30328, Santa Cruz), phosphor-Stat3 (9213, Cell Signaling), Stat3 (sc-482, Santa Cruz), Lamin B (sc-6216, Santa Cruz) and β-actin (sc-7778, Santa Cruz) antibodies. The membranes were blocked with blocking solution (5% skim milk in TBS; 50 mM Tris-base, pH 7.4, 0.15 M NaCl, and 0.1% Tween-20) for 1 h, and incubated with primary antibodies in blocking solution for 16 h. The membranes were washed three times for 10 min in TBS and then incubated with HRP-conjugated anti-mouse or anti-rabbit antibodies (0.1 Ag/mL) for 1 h. Immunoreactivity was detected by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ; http://www.amersham biosciences.com).

Immunofluorescence staining

MESCs were cultured on gelatin-coated cover slips. After washing twice with PBS, cells were fixed with 4% paraformaldehyde for 15 min. The cover slips were washed three times with PBS and the cells were permeabilized with 0.1% Tween-20 in PBS for 20 min followed by blocking for 30 min using blocking buffer (5% bovine serum albumin in PBS). After overnight incubation with the primary antibodies, the cover slips were washed three times with PBS and treated with Alexa Fluor 488 donkey anti-mouse IgG (Cat No: A21202, Invitrogen) or Alexa Fluor 594 donkey anti-rabbit IgG (Cat No: A21207, Invitrogen) for 1 h in the dark. The cover slips were then washed three times in PBS and mounted with VECTASHIELD Mounting Medium with DAPI (Cat No: H-1200, Vector Laboratories, Burlingame, CA, USA; http://www.vectorlabs.com). Images were captured using an inverted microscopy system (ECLIPSE E600; Nikon, Kanagawa, Japan).
Expression of Ell3 in mouse ESCs

In a previous study, we reported that comparing the gene expression profiles of oocytes and ESCs with those of differentiated cells is a valuable approach to identifying novel factors involved in the regulation of self-renewal or pluripotency of ESCs [13]. Comparison of the immature oocyte specific transcriptome, which was previously obtained using the annealing control primer polymerase chain reaction (ACP-PCR) technique [22], with that of mESCs revealed that both oocytes and mESCs express Ell3, a testis-specific RNA polymerase II elongation factor. As shown in Fig. 1A, Ell3 is actively expressed in mESCs, but the transcripts are weakly detected in differentiated cells such as mouse embryonic fibroblasts (MEFs) and NIH3T3 cells. These results suggest that Ell3 may be confined to the undifferentiated state of mESCs. To test this idea, the expression level of Ell3 was analyzed in mESCs treated with retinoic acid (RA) to induce differentiation. Surprisingly, expression of Ell3 transiently increased during EB formation and in the early stages of spontaneous differentiation (up to 4 days), but subsequently decreased as differentiation progressed (Fig. 1B), suggesting that Ell3 may play a role in the early differentiation of mESCs. To investigate the function of Ell3 in mESCs, stable Ell3-OE or KD mES cell lines were generated. Analysis of Ell3 mRNA in the OE or KD cell lines confirmed that the expression level of Ell3 was stably maintained (Fig. 1C).

Ellifit and its cognate signaling pathway through Jak/Stat3 are crucial for self-renewal and pluripotency in mESCs [13,14]. Phospho-Stat3 levels were therefore examined in Ell3-OE and KD cell lines. As shown in Fig. 1D, the phospho-Stat3 levels in Ell3-OE or KD cell lines were similar to those of control cells. In addition, the level of Oct4, a self-renewal marker of ESCs, was not affected by the change in Ell3 expression. These results indicate that changes in Ell3 levels do not affect the expression of major factors governing self-renewal of mESCs.

The efficiency of secondary EB formation, which reflects the capacity of ESCs to maintain an undifferentiated state and self-renewal capacity [23], was examined next. Surprisingly, the efficiency of secondary EB formation in Ell3-OE cells was 50% higher than that in control cells, while in Ell3-KD cells the secondary EB formation efficiency was lower than that in control cells when measured 10 days after EB formation (Fig. 1E). This result correlates with the finding that Ell3 expression increases during EB formation (Fig. 1B) and strongly suggests that the expression level of Ell3 affects the efficiency of EB formation, even though it does not regulate Stat3 signaling or the expression of self-renewal markers.

Ell3 regulates pluripotent differentiation of mESCs

The morphology of Ell3-OE and KD mESCs was indistinguishable from that of control cells under self-renewal or EB forming conditions (Fig. 2A). As in control cells, Ell3 expression levels in Ell3-OE and KD cells increased as mESCs differentiated into EB or underwent RA-induced differentiation (Fig. S1). However, Ell3-OE cells differentiated more rapidly than control cells, while Ell3-KD cells were resistant to differentiation and showed a cell death phenotype when exposed to RA (Fig. 2A). The expression of lineage markers such as nestin, gata4, and brachyury-T was markedly increased in EBs or differentiated Ell3-OE cells compared to control cells, whereas the decrease of self-renewal marker expression was similar between Ell3-OE and control cells (Fig. S2). We then examined whether suppression of Ell3 expression could inhibit the enhanced differentiating capacity of Ell3-OE cells. Indeed, transfection of siRNA targeting Ell3 attenuated the enhanced differentiation capacity of Ell3-OE cells (Fig. 2B).

Despite the enhanced differentiation potential of Ell3-OE cells, the expression of self-renewal factors such as Oct4, Sox2 and Nanog during differentiation decreased in similar levels in control and Ell3-OE cells (Fig. 2C). In addition, Stat3 and p-Stat3 were also decreased to a similar extent in the differentiating control and Ell3-OE cells (Fig. 2D). These results suggest that the enhanced differentiation of Ell3-OE cells is not associated with alterations in the self-renewal capacity of mESCs.

To confirm that Ell3 plays a role in pluripotent differentiation in vivo, Ell3-OE or KD mESCs were injected into BALB/c nude mice, and teratoma formation was monitored. As shown in Fig. 2E, teratoma development occurred more rapidly in Ell3-OE cell-injected animals than in controls, while Ell3-KD cell-injected mice did not develop teratomas until 7 weeks following transplantation. When examined by histological staining, teratomas harvested 4 weeks after transplantation of Ell3-OE cells showed well-developed tissues consisting of all three germ layers: respiratory epithelium (endoderm), muscle (mesoderm), and epidermis (ectoderm) (Fig. 2F, left panel). By contrast, teratomas from Ell3-KD cells did not show the typical staining of specific lineage cell types (Fig. 2F, right panel). Taken together, these results indicate that Ell3 regulates the pluripotent differentiation of mESCs.

The effect of Ell3 expression on the neural differentiation of mESCs

To further investigate the effect of Ell3 on lineage specific differentiation, we compared the neural differentiation of Ell3-OE cells to that of control cells. Compared with control cells, Ell3-OE cells rapidly lost ESC morphology within 3 days in neural induction media and showed significantly enhanced differentiated
morphology during differentiation (Fig. 3A). Ell3 expression increased during neural differentiation both in control and Ell3-OE cells (Fig. S3). Quantitative RT-PCR analysis of Ell3 in ESCs, EBs, and differentiated cell stages (RA-D4, RA-D8, RA-D12 and RA-D16). Ell3 transcripts increased at the EB and RA-D4 stages, and subsequently decreased as differentiation progressed (RA-D8, RA-D12 and RA-D16). (C) Ell3 transcript levels in Ell3-OE and KD cells at passage 10 were compared with those in control cells. Passage was counted after Ell3-OE and KD stable cell lines were established. (D) Expression of Stat3, p-Stat3, and Oct4 in Ell3-OE and KD cells was compared with that in control mESCs. β-actin was used as a loading control for immunoblot analysis. (E) Primary EBs of Ell3-OE, Ell3-KD, and control mESCs were dissociated into single cells and re-seeded at a density of 1 × 10⁶ cells/mL in the same medium. The number of secondary EBs was counted under a bright microscope (n > 3). All experiments were performed at least in triplicate, and all values represent the mean ± s.d. from at least triplicate experiments. *Indicates significant (P < 0.05) results (Student's t-test).

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Figure 1. Ell3 is specifically expressed in mESCs. (A) RT-PCR and real-time RT-PCR analysis shows that Ell3 is expressed in mESCs but not in MEF and NIH3T3 cells. (B) Quantitative RT-PCR analysis of Ell3 in ESCs, EBs, and differentiated cell stages (RA-D4, RA-D8, RA-D12 and RA-D16). Ell3 transcripts increased at the EB and RA-D4 stages, and subsequently decreased as differentiation progressed (RA-D8, RA-D12 and RA-D16). (C) Ell3 transcript levels in Ell3-OE and KD cells at passage 10 were compared with those in control cells. Passage was counted after Ell3-OE and KD stable cell lines were established. (D) Expression of Stat3, p-Stat3, and Oct4 in Ell3-OE and KD cells was compared with that in control mESCs. β-actin was used as a loading control for immunoblot analysis. (E) Primary EBs of Ell3-OE, Ell3-KD, and control mESCs were dissociated into single cells and re-seeded at a density of 1 × 10⁶ cells/mL in the same medium. The number of secondary EBs was counted under a bright microscope (n > 3). All experiments were performed at least in triplicate, and all values represent the mean ± s.d. from at least triplicate experiments. *Indicates significant (P < 0.05) results (Student's t-test).

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Activation of Zeb1 expression is the major cause of enhanced differentiation of Ell3-overexpressing mESCs

Recently, it was shown that EMT is an early and essential step in lineage specification of ESCs [9]. To investigate the underlying mechanism of Ell3 promotion of mESC differentiation, we compared the gene expression patterns of Zeb1 and Zeb2, core transcription factors that induce EMT, between Ell3-OE and control cells. As expected, the expression of Zeb1 and Zeb2 was significantly higher in Ell3-OE cells than in control cells, both in self-renewal and differentiating stages (Fig. 4A). Since Zeb factors are core transcriptional repressors that suppress the expression of epithelial genes, including E-cadherin, as well as stemness-inhibiting microRNAs [24,25], we analyzed the expression of E-cadherin to examine whether EMT was increased during the differentiation of Ell3-OE cells. As expected, immunoblot analysis showed that E-cadherin expression in differentiating Ell3-OE cells was lower than that in control cells (Fig. 4B). In contrast to E-cadherin, N-cadherin induces invasion, migration, and EMT of multiple cancer cell lines [26,27]. Consistently, N-cadherin and other EMT markers such as Mmp9 and Mmp25 were significantly expressed during the differentiation of Ell3-OE cells (Fig. 4C). These results suggest that the enhanced differentiation capacity of Ell3-OE cells may be due to the rapid induction of EMT.
Next, we analyzed whether Zeb1 and Zeb2 are downstream targets of Ell3 in mESCs. MESCs were transfected with siRNAs targeting Zeb1 or Zeb2 and examined for phenotypic changes. Interestingly, the enhanced differentiation capacity of Ell3-OE cells was compromised by the transfection of Zeb1 siRNA, whereas suppression of Zeb2 did not affect the differentiation of mESCs (Fig. 4D). Consistently, the expression of Nestin, which was enhanced during the differentiation of Ell3-OE cells (Fig. 3B), was also suppressed by the knockdown of Zeb1 (Fig. 4E). However, knockdown of Zeb2 in Ell3-OE cells did not suppress the enhanced expression of Nestin. Taken together, these results suggest that Zeb1, but not Zeb2, is a downstream target of Ell3 that induces EMT during the differentiation of mESCs.

Apoptosis of differentiating Ell3 KD is associated with enhanced p53 expression and caspase pathway activation

As shown in Fig. 2A, differentiating Ell3-KD cells showed a cell death phenotype. When we analyzed the cell cycle, we found that the sub-G1 population in differentiating Ell3-KD cells was significantly increased compared with that of the control cells (Fig. 5A). In addition, Annexin V/PI staining showed increased cell death in differentiating Ell3-KD cells compared with control cells (Fig. 5B). As caspases are key molecules in apoptosis, the possible relationship between caspase activation and cell death of Ell3-KD cells was estimated by analyzing the amount of Lamin B, a proteolysis substrate for activated caspase-3 and -6 [28,29]. Immunoblotting results showed complete degradation of Lamin B protein in differentiating Ell3-KD cells, confirming increased activity of the caspase pathway in Ell3-KD cells compared with control cells during differentiation (Fig. 5C). To confirm that apoptosis in differentiating Ell3-KD was indeed caused by Ell3
suppression, we ectopically transfected an Ell3-expressing plasmid into Ell3-KD cells and induced differentiation. Expectedly, introduction of an Ell3-expressing plasmid into Ell3-KD cells prevented apoptotic cell death and induced differentiation comparable to that of control cells in differentiation media (Fig. 5D). Apoptosis of Ell3-KD cells under differentiation conditions significantly decreased with the re-expression of Ell3 (Figure 5E). To confirm that activation of the caspase pathway during the differentiation of mESCs depends on Ell3 expression, the amounts of Lamin B, procaspase-3, and procaspase-9 were analyzed after the forced expression of Ell3 in Ell3-KD cells. As shown in Fig. 5F, Ell3-KD cells transfected with an Ell3-expressing plasmid expressed higher amounts of Lamin B, procaspase-3, and procaspase-9 3 days after RA-induced differentiation, which indicates that forced expression of Ell3 in Ell3-KD inhibited activation of the caspase pathway in differentiated mESCs. These results indicate that Ell3 regulates caspase-dependent apoptosis in mESCs during differentiation.

Since the C-terminal domain of Ell3 shows strong similarities to that of Ell, which acts as a negative regulator of p53 [20,21] (a major mediator of apoptosis in mammalian cells), we investigated whether Ell3 expression affects the amount of p53 in differentiating mESCs. Indeed, the p53 protein level, which was significantly higher in differentiating Ell3-OE cells compared with control cells, returned to the control cell level when Ell3-KD cells were transfected with an Ell3-expressing plasmid (Fig. 5G). These results suggest that Ell3 functions as a negative regulator of p53 in differentiating mESCs. Consistently, p53 expression in differentiating Ell3-OE cells was significantly lower than that in control cells, and depletion of Ell3 by siRNA resulted in an increase in p53 protein levels (Fig. 5H, Fig. S4A). Furthermore, Ell3 siRNA-mediated depletion of Ell3 enhanced apoptosis in Ell3-OE cells during differentiation (Fig. S4B). These results show that changes in p53 protein expression in differentiating Ell3-OE or KD mESCs depend on the expression level of Ell3.

Discussion

Our data establish a model whereby Ell3 promotes EMT and suppresses p53 levels, which leads to the initiation of differentiation of mESCs. We showed that Ell3 overexpression promotes differentiation of mESCs with the concomitant activation of EMT marker genes. In addition, Ell3-knock-down mESCs undergo apoptosis along with an accumulation of p53. Interestingly, we found that the activation of Zeb1, which is known to link EMT-activation and stemness maintenance in mESCs [24], is an essential event for Ell3 to promote differentiation of mESCs, as shown by the finding that suppression of Zeb1 in Ell3-OE cells compromises the differentiation-promoting effect of Ell3. Based on
these results, we propose that the promotion of EMT may account for the role of Ell3 in ESC differentiation, which is in line with a previous study demonstrating that EMT is an early and essential step in the differentiation of ESCs [9]. Since Ell3 is known as a transcription elongation factor, it would be important to elucidate whether Ell3 directly regulates the expression of Zeb1 and Zeb2. The mechanism by which Ell3 activates EMT marker genes, including Zeb1 and Zeb2, is currently under investigation in our laboratory.

Another important advance of this study is the discovery that p53 protein stability is enhanced, and the caspase pathway is activated, when Ell3 expression is suppressed in differentiating mESCs. P53 functions as a decision maker in mESCs, inducing differentiation by repressing Nanog expression, [30] or inhibiting differentiation by inducing expression of several WNT ligands [31]. In vitro differentiation of mESCs results in decreased levels of p53 and shifts p53 conformational status to the mutant form, allowing differentiating cells to evade apoptosis [32]. Our study indicates that Ell3 may function to safeguard differentiating ES cells by escaping apoptosis via suppression of p53.

Ell3 overexpression or suppression did not affect the protein or transcript levels of p53 in the self-renewal state of mESCs, indicating that Ell3-mediated regulation of p53 was active only under differentiation conditions (Fig. S3). One possible hypothesis is that other factors induced during mESC differentiation may cooperate with Ell3 to activate the p53 degradation pathway when mESCs transition from self-renewal to differentiation. Additional studies are needed to elucidate how Ell3 only affects p53 expression in differentiating mESCs, but not in self-renewing mESCs.

Activation of caspase-3 induces differentiation of ESCs by inducing the cleavage of Nanog [33]. However, the enhanced activity of caspase-3 in Ell3-KD cells induced apoptosis instead of promoting differentiation. This result indicates that a more complex mechanism may underlie the involvement of caspase activity in the differentiation of ESCs. One possibility is that caspase levels may be regulated to balance the rates of differentiation and apoptosis in ESCs during differentiation.

The regulatory mechanism underlying how Ell3 regulates p53 expression remains elusive. Ell3 did not have an affect on p53 transcription, suggesting that Ell3 controlled p53 levels by modulating p53 protein stability (data not shown). Since Mdm2-mediated ubiquitination-dependent degradation is one of the main pathways negatively regulating p53 levels, future studies should...
examine whether Ell3 regulates the level of p53 by the Mdm2-mediated ubiquitination pathway.

It remains unclear whether there is a link between the regulation of EMT and that of p53 expression by Ell3. Indeed, p53 loss of function or mutations was recently found to promote cancer cell EMT by de-repressing Snail 1 protein expression and activity [34]. Therefore, it would be interesting to study the link between p53 and EMT in the initiation of ESC differentiation.

Supporting Information

Figure S1 Ell3 transcripts in Ell3-OE and KD cells during EB formation or RA-induced differentiation were quantitatively compared with those in control cells. Five-day-old EBs (EB-D5) or cells differentiated for 3 days (RA-D3) were used for the analysis.

Figure S2 (A) Oct4, Sox2, and Nanog expression in Ell3-OE and control mESCs was analyzed by real-time RT-PCR 0, 3, 5, and 7 days after RA-induced spontaneous differentiation. (B) Nestin, Gata4, and Brachyury-T expression in Ell3-OE and control mESCs was analyzed by real-time RT-PCR in 5 days old EBs (EB-D5) or in RA-induced differentiated cells (RA-D3).

Figure S3 Expression level of Ell3 during the neural differentiation of Ell3-OE or control mESCs was analyzed by real-time RT-PCR.

Figure S4 Ell3-OE cells were transfected with non-specific siRNA (siNS) or Ell3-targeting siRNA (siEll3) for 48 h. Ell3 transcript levels were compared with those in

Figure 5. Differentiating Ell3-KD cells undergo apoptosis, which is associated with enhanced p53 expression and activated caspase pathway. (A) Cell cycle distribution of control and Ell3-KD cells stained with propidium iodide (PI). Cells in the ESC state or differentiated for 3 days (RA-D3) by removing LIF and adding retinoic acid (RA) were analyzed by flow cytometry. (B) Apoptosis of control and Ell3-KD cells was quantitatively analyzed either in the ESC or differentiated state by determining the number of Annexin V-positive cells. Cells were spontaneously differentiated for 3 days (RA-D3). (C) The amounts of Lamin B in control or Ell3-KD cells were determined either in the ESC state or in spontaneously differentiated cells for 3 days (RA-D3) by immunoblot analysis. β-actin was used as a loading control. Control (V) or Ell3-expressing vectors (Ell3) were transfected into Ell3-KD cells in the ESC state, and transfected cells were spontaneously differentiated for 3 days. Cells were examined under the microscope (D) and apoptosis was quantitatively analyzed by determining the number of Annexin V-positive cells (E). (F) The amounts of Lamin B, procaspase-3, and procaspase-9 in Ell3-KD cells transfected with control or Ell3-expressing plasmids were determined either in the ESC state or in spontaneously differentiated cells after 3 days by immunoblot analysis. β-actin was used as a loading control. (G) Ell3-KD cells were transfected with control (V) or Ell3-expressing plasmids (Ell3). Transfected cells were spontaneously differentiated for 3 days, and p53 levels were examined by immunoblot analysis. β-actin was used as a loading control. (H) p53 in control or Ell3-OE cells was determined after 3 days of spontaneous differentiation by immunoblot analysis (left panel). Ell3-OE cells were transfected with nonspecific siRNA (siNS) or Ell3-targeting siRNA (siEll3). Transfected cells were spontaneously differentiated for 3 days, and p53 was examined by immunoblot analysis (right panel). β-actin was used as a loading control. All experiments were performed at least in triplicate and all values represent the mean ± s.d. from at least triplicate experiments. * Indicates significant (P<0.05) and ** highly significant (p<0.01) results (Student’s t-test).

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control cells transfected with siNS (A), and apoptosis was quantitatively analyzed by determining the number of Annexin V-positive cells (B). All values represent the mean ± s.d. from at least triplicate experiments. ** Indicates highly significant (P<0.01) results (Student’s t-test).

(TIF)

**Figure S5** RNA or protein levels of p53 in Ell3-OE or control mESCs were analyzed by real-time RT-PCR or immunoblot analysis.

(TIF)

**Table S1** Real time PCR primer sequences used in this study.

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(TIF)

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**Author Contributions**

Conceived and designed the experiments: HA KP. Performed the experiments: HA YC YM JJ. Analyzed the data: HA KP. Contributed reagents/materials/analysis tools: YC. Wrote the paper: KP.