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Mouse embryonic stem cells resist c-Jun induced differentiation when in suspension

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ABSTRACT

The oncogene *c-Jun* plays a key role in development and cancer. Yet, its role in cell fate decision remains poorly understood at the molecular level. Here we report that c-Jun confers different fate decisions upon mouse embryonic stem cells (mESCs) in adhesion vs suspension culture. We developed a Tet-on system for temporal induction of c-Jun expression by Doxycycline treatment in mESCs. We show that mESCs carrying the inducible c-Jun TetOn remain pluripotent and grow slowly in suspension when c-Jun expression is induced, whilst when the cells adhere they undergo differentiation and show normal proliferative potential upon c-Jun induction. Our data indicates that c-Jun pushes mESCs in suspension into cell cycle arrest at G1/S, by activating the cell cycle inhibitors Cdkn1a/b and Cdkn2/a/b/c. Despite this cell cycle arrest, they can still re-enter the cell cycle upon transfer to an adhesive surface, and grow into typical mESC colonies, albeit at a lower efficiency. These results demonstrate that mESCs respond to induced c-Jun overexpression differently in suspension or adherent cultures. Our results suggest that cells in suspension may be more resistant to differentiation than when they adhere.

1. Introduction

c-Jun was the first oncogene identified as a transcription factor, highlighting the link between oncogenesis and transcription regulation.[1–4] It contains a leucine zipper domain that can bind to cis-acting DNA elements found frequently in promoters of genes responsive to growth factors or other stimuli.[5,6] Mice lacking c-Jun fail to develop and die around E10.5, suggesting that c-Jun plays a critical role in cell fate decisions during development.[7–9] Interestingly, we have shown that overexpression of c-Jun blocks the generation of induction of pluripotent stem cells (iPSCs) from mouse embryonic fibroblasts (MEFs) by preventing a mesenchymal to epithelial transition (MET)[10] and skewing the chromatin accessibility dynamics.[11] During somatic cell reprogramming, c-Jun indeed not only inhibits the MET process, but also blocks the reactivation of epithelial and pluripotent genes such as *Cdh1, Epcam, Nanog* and *Esrrb*. Similarly, inhibition of c-Jun accelerates the reprogramming process.[10] Recent studies have provided a comprehensive molecular analysis of the reprogramming process,

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reporting that c-Jun binding partner Fra1 is critical for somatic program silencing and the repression of Fra1 is required for E-cadherin (Cdh1) activation.[12] Those reports suggest that the AP-1 family of transcription factors, such as c-Jun and Fra1 play an important role in cell fate decisions.

As a member of AP-1 family, c-Jun regulates a wide range of cellular processes, including cell cycle, proliferation and cell adhesion.[13–16] Studies carried out many years ago demonstrated that c-Jun is required for proliferation. c-Jun$^{-/-}$ embryonic fibroblast cells exhibited a defect for progression through the G1 phase of the cell cycle. The low expression level of cyclin D1 was identified as partially responsible for this cell cycle defect.[15] Another study reported the expression of cyclin D1 mediated by c-Jun as a key events during cell transformation upon arsenite exposure.[16] c-Jun and other AP-1 family TFs are closely associated with c-Jun N-terminal kinase (JNK)-mediated proliferation in fibroblast growth factor-4 (FGF4)-stimulated mESCs.[13] mESCs lacking c-Jun exhibit a defect in differentiation into hepatocytes and overexpression of c-Jun results in exit from pluripotency. Mechanistically, c-Jun regulates mESC differentiation by controlling the expression of genes associated with cell adhesion such as Fn1 (Fibronectin 1).[14] c-Jun was also reported to regulate the expression of adhesion molecules such as NCAM during injury response.[17]

It has been proposed that c-Jun acts as a guardian of somatic fate, opposing the pluripotency network in ESCs.[10] In this report, we show that c-Jun can be compatible with pluripotency, but only under suspension culture conditions, and by inducing cell cycle arrest.

2. Results and discussions

2.1. c-Jun triggers no differentiation of ESCs in suspension

Previously, we developed an inducible c-Jun TetOn ESCs, which bear an Oct4-GFP reporter whose expression reflects pluripotency. By this inducible approach, we have shown that c-DOX (Doxycycline)-induced c-Jun overexpression in ESCs triggers rapid differentiation with a loss of pluripotent markers.[10] Surprisingly, when we culture c-Jun TetOn ESCs with or without DOX (2 μg/ml) for 72 h in suspension in ultra-low attachment tissue culture plates, we show that c-Jun induction did not trigger differentiation and the cells remained Oct4-GFP positive, a reporter for pluripotency (Fig. 1A), but c-Jun expression significantly impacted the colony size and proliferation of cells in suspension (Fig. 1B and C). Compared to the same cells grown in adhesion for 6 or 36 h, they lose Oct4-GFP expression totally (Fig. 1D). However, c-Jun has little effect on the proliferative potential of differentiating cells in adherent culture (Fig. 1E). These results strongly suggest that c-Jun can become compatible with the maintenance of pluripotency in suspension, but remains incompatible with pluripotency when the cells are adherent, as reported previously.[10]

2.2. c-Jun induces cell cycle arrest in ESCs in suspension

The reduction in cell number suggests that ESCs grown in suspension may undergo cell cycle arrest when c-Jun is induced by DOX (Fig. 1). To test this idea, we profiled the cell cycles of

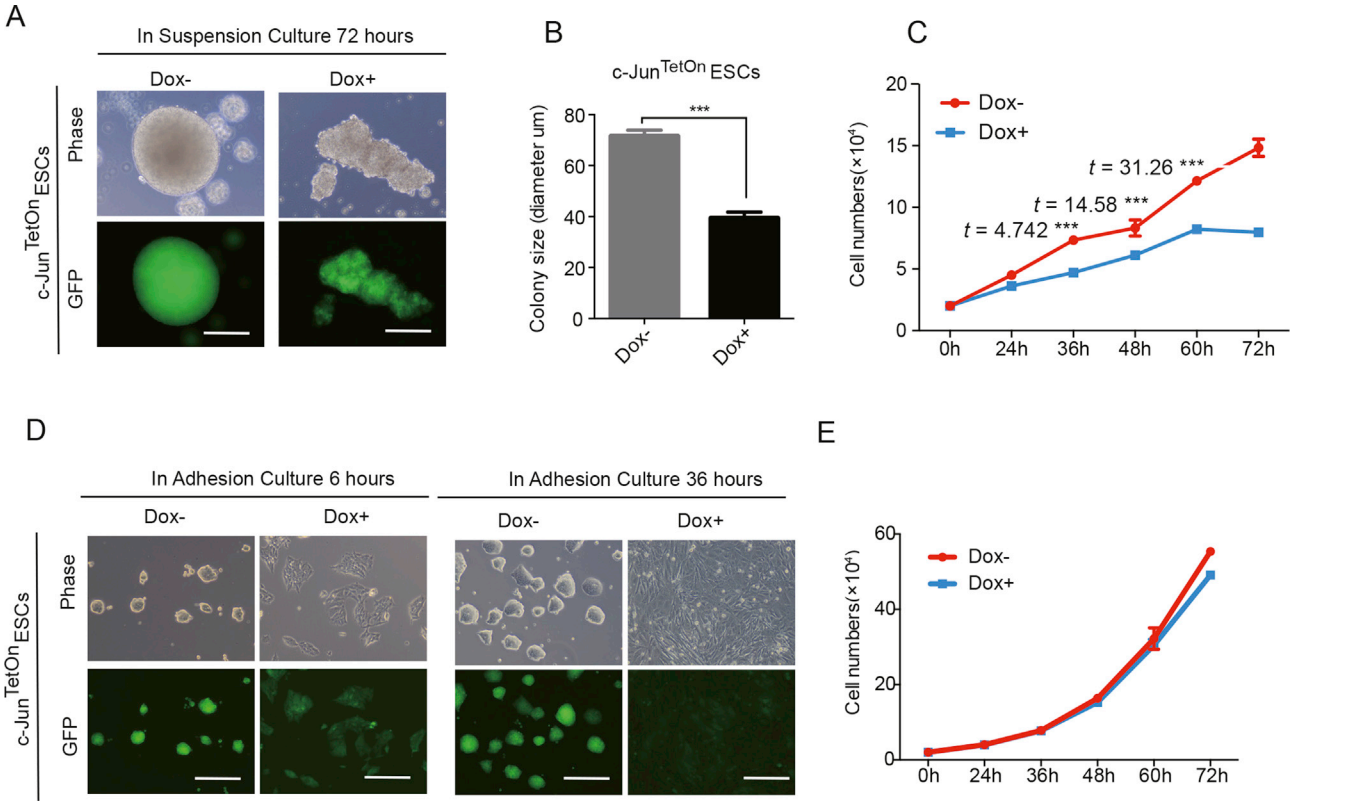

Fig. 1. c-Jun induction regulates mouse ESCs differentiation. (**A**) c-Jun induction regulates ESC differentiation in suspension culture. Scale Bar = 50 μm c-Jun TetOn ESCs were an established cell line that was co-infected by rtTA and inducible c-Jun lentiviruses. c-Jun expression can be induced by Doxycycline (DOX) treatment.[10] (**B**) Induction of c-Jun results in small size colonies formation. (**C**) c-Jun induced inhibited cell proliferation in suspension culture, (mean ± s.d., two-way ANOVA, Sidak's multiple comparisons; n = 6 wells pooled from two independent experiments, t ratios are providing for significant differences 36h***t = 4.742, 48h***t = 14.58, 60h***t = 31.26). **(D)** Induction of c-Jun causes adherent ESC cells differentiation with morphological changes in 6 h and total loss of Oct4-GFP in 36 h. Scale Bar = 50 μm. (**E**) Growth curves of c-Jun TetOn ESCs in 2i + LIF medium with DOX treatment or not (mean ± s.d., n = 6 wells pooled from two independent experiments).

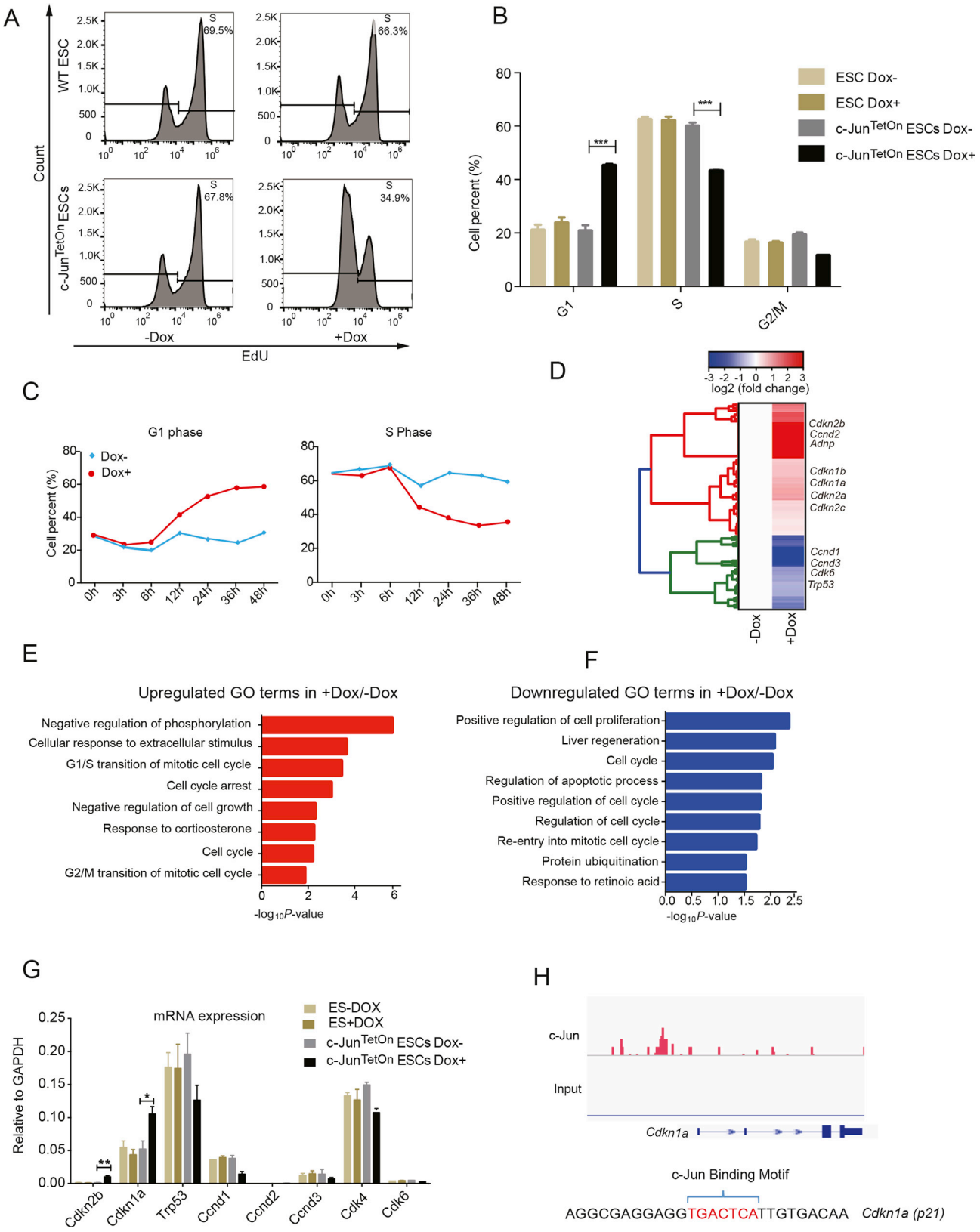

Fig. 2. c-Jun inhibits cell cycle during differentiation. (A) Induction of c-Jun suppresses cell proliferation. Cell cycle was measured by flow cytometry with EdU which could incorporate into the DNA of dividing cells. Numbers inside the plots indicate the percent of cells passing through S-phase over 12 h (B) c-Jun arrests the cell cycle at the G1/S transition. Cell cycle was measured by flow cytometry with propidium iodide staining (PI), a fluorescent molecule used to evaluate DNA content in cell cycle analysis. (C) G1 and S phase cell percentage at different time points with or without c-Jun induction. Data was collected by flow cytometry with PI staining. (D) RNA-seq analysis of c-Jun TetOn ESCs

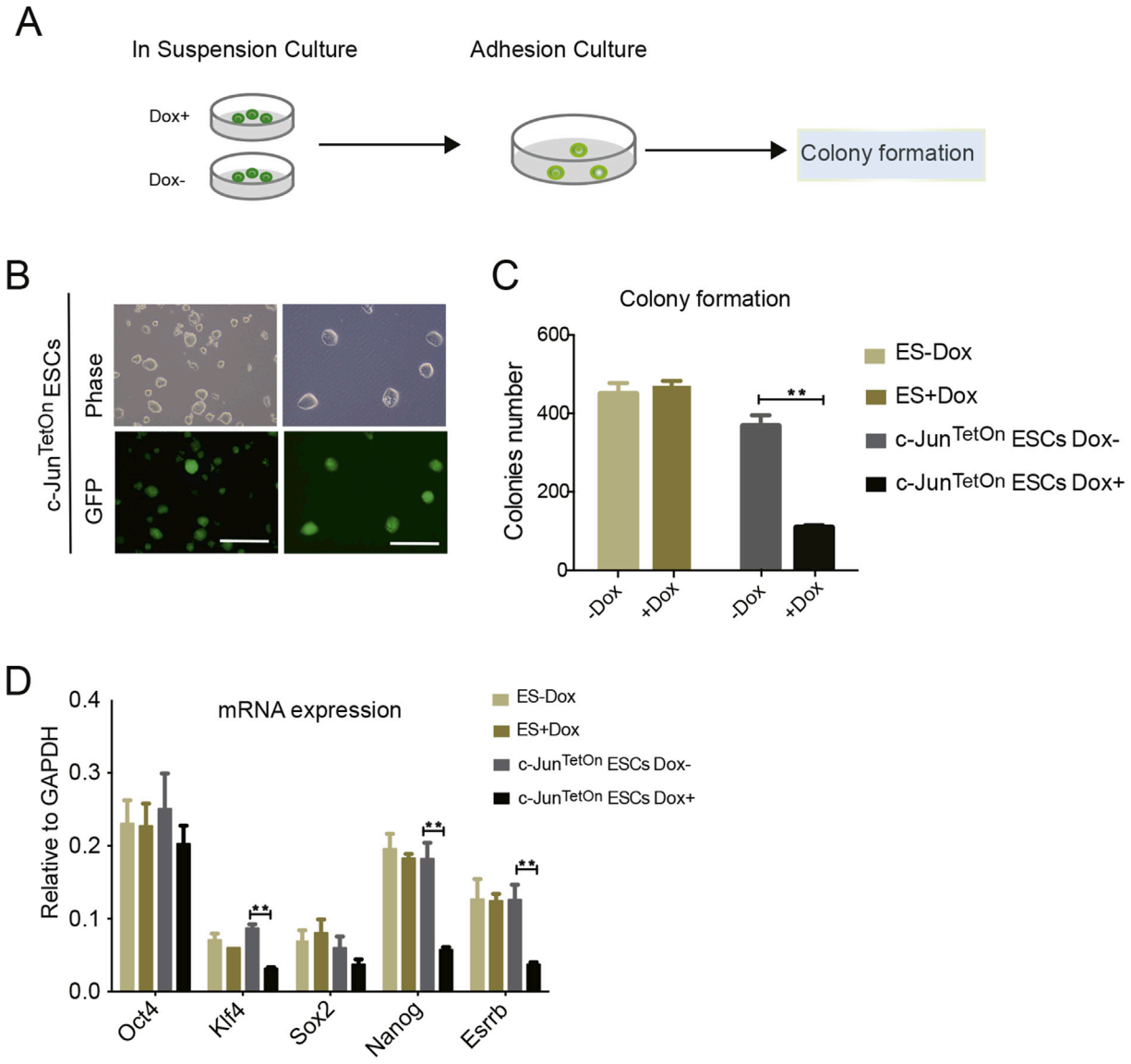

Fig. 3. c-Jun induced mouse ESC exit from pluripotency. (**A**) Schematic for colony formation analysis of re-plated c-Jun TetOn ESCs cells in adhesion after being cultured in suspension with or without DOX for 36 h. (**B**) Induction of c-Jun impeded colony formation. Scale Bar = 50 μm. (**C**) c-Jun induction inhibited colony formations. Data are from 6 biological replicates in 2 independent experiments and are shown as the mean ± SEM. *p value < 0.05, **p value < 0.01, one-way ANOVA with Dunnett's test. (**D**) The expression pattern of the respective pluripotent genes. Data are from 6 biological replicates in 2 independent experiments and are shown as the mean ± SEM. *p value < 0.05, **p value < 0.01, one-way ANOVA with Dunnett's test.

both wild type and c-Jun TetOn ESCs and show that WT ESCs are consistent with G1/S/G2/M ratios with or without DOX, while c-Jun TetOn ESCs have elevated G1 and reduced S under DOX induction, as measured by propidium iodide staining (Fig. 2A and B). In a time course experiment, we show that c-Jun induction gradually shifts G1 cells from 30% to around 60% within 48 h, with a concurrent reduction of S phase from >60% to around 30%.

treated with and without DOX in suspension culture for 36 h. The selected genes for this heatmap were enriched at least 2 fold upregulated or downregulated in DOX treated sample relative to DOX untreated sample. Red and blue indicate increased and decreased expression, respectively. A selection of cell cycle specific genes is indicated on the right of the heatmap. (E) Gene ontology analysis of the RNA-seq data obtained from c-Jun TetOn ESCs with and without DOX treatment in suspension culture for 36 h. Upregulated genes are defined in the heatmap. Analysis was performed with DAVID using EASE score (a modified Fisher exact test), *Benjamini-Hochberg corrected p value (Q value) < 0.05. (F) Gene ontology analysis of the downregulated genes defined in the heatmap, *Benjamini-Hochberg corrected p value (Q value) < 0.05. (G) The expression of cell cycle related genes of suspension cultured cells by q-PCR. Data are from 6 biological replicates in 2 independent experiments and are shown as the mean ± SEM. *p value < 0.05, **p value < 0.01, one-way ANOVA with Dunnett's test. (H) Genome view of c-Jun ChIP-seq data at the Cdkn1a locus. ChIP-seq data were obtained from ESCs that were treated with DOX for 36 h.[10] The sequence of the c-Jun (AP-1) binding motif is indicated below.

Cdkn1a and Cdkn2b are cyclin-dependent kinase inhibitors which bind to and inhibit the activity of Cdk2 and Cdk4, functions as a negative regulator cell cycle progression at G1 and S phase.[18,19] The D-type cyclins, Ccnd1 and Ccnd3 form a complex with, and function as regulatory subunits of Cdk4 and Cdk6, whose activity is required for the cell cycle G1/S transition.[20–22] The apparent cell cycle arrest caused by induction of c-Jun, correlated with the activation of Cdkn1a, Cdkn2b and the down-regulation of Ccnd1, Ccnd3, Cdk6 (Fig. 2D). Consistently, GO analysis of down-regulated genes indicated they belong to cell cycle regulation biological processes (Fig. 2E and F). q-PCR analysis confirmed the up regulation of Cdkn2b and Cdkn1a (Fig. 2G). According to c-Jun ChIP-seq data, we found c-Jun binding at the promoter region of Cdkn1a, suggesting that Cdkn1a was regulated by c-Jun directly (Fig. 2H).[16,20,23,24]

2.3. c-Jun arrested ESCs in suspension can reenter the cell cycle when allowed to adhere

To see if the cell cycle arrested ESCs can re-enter cell cycle, the c-Jun TetOn ESCs were treated with or without DOX for 36 h in suspension, then the cells were digested to break up clusters, and equal numbers of single cells were re-plated on 0.1% gelatin coated adherent tissue culture plates without DOX treatment (Fig. 3A). As shown in Fig. 3 B, Cell morphology and Oct4-GFP indicated that the cells did not lose pluripotency, and could proliferate and form colonies. Cell colony formation was evaluated by counting the number of colonies derived from single cells after culturing on adherent plates without DOX for a further 72 h. The replated single cells can grow into typical ESC colonies independent of DOX treatment, albeit the c-Jun induced mESCs resulted in reduced numbers. While they maintain the expression of Oct4 at a normal level, we can detect a reduction of Klf4, Sox2, Nanog and Esrrb in DOX induced ESCs (Fig. 3D). Nonetheless, these cells can re-enter a normal cell cycle, and maintain pluripotency when switched to adhesive culture conditions.

3. Conclusion

We have shown here that overexpression of c-Jun can be compatible with pluripotency, but only in suspension, this is in sharp contrast to our earlier findings that c-Jun induces rapid differentiation of ESCs in adhesion culture and impedes the acquisition of pluripotency through reprogramming with Yamanaka factors.[10] Interestingly, c-Jun induction in mESCs results in both a reduced colony size and a corresponding cell number reduction when cultured in suspension. The mESCs grown in suspension apparently circumvent the incompatibility with pluripotency by undergoing cell cycle arrest triggered by c-Jun. This effect is mediated by the upregulation of the G1/S transition cell cycle inhibitors Cdkn1a and Cdkn2b, which then block cell cycle progression upon c-Jun induction. The arrest of cell cycle may reflect an adaptive strategy for c-Jun mESCs to negotiate cell fate decisions with the pluripotency network and may be relevant in other cell fate decision processes.

4. Methods

4.1. Cell culture

Mouse embryonic stem cells (ESCs) were derived from 3.5 d.p.c mouse embryos by crossing male Oct4—GFP (OG2) reporter allele-carrying mice[25] (CBA/CaJ X C57BL/6J) to 129Sv/Jae female mice. c-Jun TetOn ESCs were co-infected by rtTA and inducible c-Jun lentiviruses.[10] Wild type ESCs and c-Jun TetOn ESCs were cultured on feeder free plates with mES+2iL medium (DMEM, 15%FBS, NEAA, GlutaMAX, PD0325901, Chir99021, LIF) at 37 °C with 5% CO_2. Mouse ESCs cultured on 0.1% gelatin-coated plates were incubated with 0.05% trypsin for about 2 min at 37 °C until the colonies were completely dissociated into single cell. For the adhesion culture, 5×10^4 cells were seeded on 0.1% gelatin-coated plates. For wild type and c-Jun TetOn ESCs culture in suspension, ultra-low attachment plates were used. After digestion, 5×10^4 embryonic stem cells per well (P6) were plated. For c-Jun induction, medium with DOX (2 μg/ml) was used. Data was collected at day 3.

4.2. Colony size detection

Colony images with scale bars obtained by ZEN software (ZEISS). Diameter of individual Colonies was measured by Adobe Illustrator control to scale bar. All data was analyzed using the prism software.

4.3. q-PCR

Total RNAs were prepared with TRIzol. For quantitative PCR, cDNAs were synthesized with ReverTra Ace (Toyobo) and oligo-dT (Takara) and PCR reaction were performed with SYBRPremix Ex Taq Kit (TAKARA, Japan) on the ABI7300 Real-Time PCR System. Relative gene expression level was determined by the delta delta Ct method using the *Gapdh* gene as a reference. TruSeq RNA Sample Prep Kit (RS-122-2001, Illumina) was used for library constructions and sequencing done with Miseq Reagent Kit V2 (MS-102-2001, Illumina) for RNA-seq. q-PCR primers are listed in Supplementary Table 1.

4.4. Cell cycle analysis

According to the manual of a 5-ethynyl-2′-deoxyuridine (EdU) labeling/detection kit (Ribobio, Guangzhou, China), 50 μM EdU labeling medium was added to the cell culture to allow incubation for 12 h at 37 °C under 5% CO2. Afterwards, cultured ESCs were fixed with 4% paraformaldehyde (pH 7.4) for 30 min and incubated with glycine for 5 min. After wash with PBS, staining with anti-EdU working solution was performed at room temperature for 30 min. Following wash with 0.5% TritonX-100 in PBS, the cells were incubated with 5 μg/ml Hoechst 33342 dye at room temperature for 30 min, followed by observation under a confocal laser scanning microscope (TCS SP2, Leica Microsystems, Germany). The percentage of EdU-positive cells was calculated from five random fields in three wells.

Cell cycle analysis was performed by propidium iodide (PI) staining. After trypsinization, cells were fixed in 70% ethanol and incubated on ice for 15 min. Wash cells by centrifugation in PBS buffer contains 1% FBS, then discard wash buffer and permeabilized cells by adding PBS contain 0.1% TritonX100 for 5—10 min. Wash cells by centrifugation and then labeled with propidium iodide (PI)/RNase staining solution (#4087, CST), and further incubated for 15 min at room temperature. Finally, cells were analysis using Fortessa (BD). Data analysis was performed using FlowJo 7.6 (Tree Star). Histograms were visualized by GraphPad Prism 5.0.

Data availability

RNA-sequencing data described in this study was deposited with gene expression omnibus with the accession number GEO: GSE114381.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

J.L. D.L designed the experiments, and analyzed the data. B.W performed experiments J.C analyzed the data, D.P. supervised the whole study. D.P. conceived the whole study, wrote the manuscript, and approved the final version.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cr.2018.05.002.

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