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Positive regulation of additional sex comb-like 1 gene expression by the pluripotency factor SOX2

Myengmo Kang [a], Sang-Wang Lee [a], Eun-Joo Kim [b], Soo-Jong Um [a,*]

[a] Department of Bioscience & Biotechnology, Institute of Bioscience, BK21 Graduate Program, Sejong University, Seoul 143-747, Republic of Korea
[b] Department of Molecular Biology, BK21 Graduate Program, Dankook University, Gyeonggi-do 448-701, Republic of Korea

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ABSTRACT

Additional sex comb-like 1 (ASXL1) has been suggested to be an enhancer of trithorax and polycomb proteins, and functions as a dual co-regulator of retinoid acid (RA) signaling. However, the mechanism by which *ASXL1* gene expression is regulated remains unresolved. Concomitant downregulation of both SOX2 and ASXL1 during the RA-induced differentiation of P19 cells prompted us to investigate the role of SOX2 in the regulation of *ASXL1*. Knockdown of SOX2 in SOX2-rich NT2 cells resulted in the reduction of *ASXL1* expression, whereas SOX2 overexpression in SOX2-deficient H1299 cells increased *ASXL1* expression. Using a cloned *ASXL1*–luciferase reporter, we demonstrated that SOX2 directly transactivates the *ASXL1* promoter. Serial deletion and mutation studies mapped the SOX2 response element region in the *ASXL1* promoter to −1600 to −1400 bp. We showed by chromatin immunoprecipitation assay that SOX2 directly binds to the *ASXL1* promoter region. Finally, formation of embryonic bodies by ASXL1-depleted murine E14TG2a embryonic stem cells was significantly impaired, similar to SOX2-knockdown cells. From these results, we suggest that *ASXL1* may be a direct target of SOX2 and may play a role in maintaining the pluripotency of stem cells.

1. Introduction

Additional sex comb-like protein (ASXL) was first identified in *Drosophila* and is a member of the enhancer of trithorax and polycomb gene group [1,2]. Three paralogs (ASXL1, ASXL2, and ASXL3) have been identified in mammals [3–5]. Among the ASXL family members, ASXL1 is often mutated in humans, and has been linked to chronic myelomonocytic leukemia, myelodysplastic syndromes, and Bohring–Opitz syndrome [6–8]. Expression of Asxl1 in mice begins at an early embryonic stage and decreases as development progresses. No transcription factor controlling the expression of ASXL1 has yet been described. Our previous studies showed that ASXL1 acts as either a coactivator or corepressor for retinoid acid (RA) receptor (RAR) by recruiting different chromatin regulators in a cell type-specific manner [9,10]. RA binds to RAR and plays critical roles in vertebrate development and stem cell differentiation [11,12].

A member of the Sry-related high-mobility group box (SOX) family, the transcription factor SOX2 plays a critical role in maintaining the pluripotent state in human and mouse embryonic stem (ES) cells [13,14]. SOX2 expression during the early embryo stage indicates that SOX2 is essential for controlling developmental processes [15]. Reduction of the SOX2 level by RNA interference induces ES cells to differentiate into multiple cell types [16]. Various studies have shown that SOX2, cooperating with another transcription factor, OCT4, activates the expression of the *SOX2* gene and other target genes, including *Oct4*, *Nanog*, *Utf1*, and *Fgf4* [17,18]. Recently, a number of new SOX2 target genes have been identified through microarray and ChIP-on-CHIP analysis of ES cells [19–21]. During analysis of the function of Asxl1 in RA-induced differentiation of mouse P19 embryonic carcinoma (EC) cells, we realized that Asxl1 is downregulated together with Sox2. This finding prompted us to investigate whether ASXL1 is another SOX2 target gene. We mapped a SOX2 binding site in the human *ASXL1* promoter. Knockdown and overexpression of SOX2 resulted in down- and upregulation of *ASXL1* expression, respectively. Furthermore, the effect of *SOX2* knockdown on embryonic body formation by ES cells was similar to that of *ASXL1* knockdown. Overall, our data suggest that that *ASXL1* may be a direct target of SOX2 and may play a role in maintaining the pluripotency of stem cells.

Abbreviations: ASXL1, additional sex comb-like 1; SOX2, Sry-related HMG Box 2; WB, western blotting; β-gal, β-galactosidase; Luc, luciferase; shRNA, small hairpin RNA; KD, knockdown; ChIP, chromatin immunoprecipitation.

* Corresponding author. Fax: +82 2 3408 4334.
E-mail address: umsj@sejong.ac.kr (S.-J. Um).

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2. Materials and methods

2.1. Plasmid construction

To isolate a 5.9-kb fragment of the human *ASXL1* promoter, genomic DNA from HEK293 cells was subjected to PCR using the primers 5′-GGAGCTTGGGGTTTAAATGTTGAC-3′ (forward) and 5′-GGTGTGTGTGAGTGTGTGTGAAGA-3′ (reverse). The amplified fragment was subcloned into the pGL2 basic plasmid (Promega, Madison, WI). PCR was used to generate serially deleted promoter fragments. The SOX2 binding site in the *ASXL1* promoter was modified by incorporation of three mutated nucleotides by site-directed mutagenesis. SOX2 and OCT4 expression vectors were constructed using Flag (2×)-tagged pcDNA3 (Invitrogen, Carlsbad, CA).

2.2. Cell culture

P19 mouse embryonic teratocarcinoma cells, NTERA-2 (NT2) human embryonic carcinoma cells, and HEK293 embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from Invitrogen) in a 5% CO_2 atmosphere at 37 °C. H1299 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotic–antimycotic.

2.3. Transfection and luciferase reporter assays

HEK293 cells (2×10^5) were plated onto 12-well plates 24 h before transfection. They were transiently transfected with a pGL2 vector carrying the ASXL1 promoter (200 ng/well) and the indicated expression plasmids using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Cell lysates prepared 24 h after transfection were subjected to luciferase assays. Luciferase activity was measured using a dual luciferase assay kit and an analytical luminescence luminometer (Promega) and was normalized to CMV-Renilla luciferase activity. Activity was calculated relative to the control. For each assay, three independent experiments were performed in triplicate.

2.4. RNA isolation and real time RT-PCR

For RNA interference experiments, an sh-SOX2 plasmid was constructed using the pSilence vector (Life Technologies, Grand Island, NY) and transfected into NT2 cells. Total RNA was extracted using iso-RNA lysis reagent (5 PRIME, Hamburg, Germany) according to the manufacturer's instructions. cDNA was synthesized from 2 μg total RNA using random primers and M-MLV reverse transcriptase (Invitrogen). After reverse transcription, quantitative real-time PCR was performed using the following primer sets: ASXL1, 5′-GGTGATGCTGCCTCGAGTTGT-3′ (forward) and 5′-AAGGCATGGAAGGTCCGAGAG-3′ (reverse); and β-actin, 5′-CATGTTTGAGACCTTCAACACCCC-3′ (forward) and 5′-GCCATCTCCTGCTCGAAGTCTAG-3′ (reverse). All reactions were carried out using SYBR green mix (Toyobo, Osaka, Japan) and a CFX real-time PCR system (Bio-Rad, Hercules, CA). Relative expression levels were calculated as ratios normalized to the β-actin control.

2.5. Chromatin immunoprecipitation (ChIP)

SOX2-rich NT2 cells were cultured in 10 cm^2 dishes, while SOX2-deficient H1299 cells were transfected with the Flag-SOX2 expression vector overnight. The cells were washed with PBS and then fixed through incubation in 1% formaldehyde solution for 5 min at room temperature. The reaction was stopped with 0.125 M glycine. A ChIP assay was subsequently performed as described previously [10]. The following primers were used for qPCR: the region covering the SOX2 binding site (−1580/−1410), 5′-GCGGGGAAGACATTATTCTG-3′ (forward) and 5′-CCCTGAGAGAAAGGAGGAACT-3′ (reverse); and the nonspecific region covering exon 2 (+7936/+8072), 5′-CCAGCGGTACCTCATAGCAT-3′ (forward) and 5′-TTTGGTGTCATTGGAGCATC-3′ (reverse).

2.6. Embryonic body formation

E14TG2a mouse ES cells (1×10^7 cells per 60 mm bacterial Petri dish) were induced to differentiate in suspension in ES medium not containing LIF. After incubation for 24 h, cells became aggregated and started to form spheroid or embryonic bodies (EBs). EBs were photographed at 200× magnification at day 3 using a Leica microscope (Leica, Wetzlar, Germany) (scale bar, 100 μm). The sizes (diameters) of 50 EBs were analyzed and averaged using Leica LAS AF Lite software. EBs with diameters >20 μm were counted. Changes in average EB size were monitored at three different time points.

3. Results and discussion

3.1. ASXL1 expression is closely related to SOX2 expression

When dissecting the function of ASXL1 in RA signaling, we found that ASXL1 is downregulated together with SOX2 in murine embryonic teratocarcinoma-derived P19 cells, which differentiate into neural lineages in response to RA. Moreover, other ChIP-based studies have suggested that *ASXL1* may be a common target of self-renewal transcription factors including SOX2 [20,21]. These findings prompted us to determine whether *ASXL1* could be a SOX2 target gene. As shown in Fig. 1A, Sox2 and Asxl1 were downregulated during RA-induced differentiation of P19 cells. Differentiation into the neural lineage was monitored by increased expression of the neural marker NF160 (Fig. 1A). Similarly, ASXL1 mRNA expression was downregulated during RA treatment (Fig. 1B), suggesting that *Asxl1* is regulated at the transcriptional level. To further investigate the correlation between SOX2 and ASXL1, we measured the expression of these factors in H1299, NT2, and HEK293 human cells. In general, ASXL1 expression was higher in SOX2-rich embryonic cells (NT2 and HEK293 cells) than in H1299 lung carcinoma cells, which are SOX2-deficient and fully differentiated (Fig. 1C). To determine whether SOX2 is an upstream regulator of the *ASXL1* gene, we depleted SOX2 in NT2 cells using sh-SOX2 and found that the ASXL1 level was reduced (Fig. 1D). Finally, ASXL1 mRNA expression in H1299 cells was significantly increased by the forced expression of SOX2 (Fig. 1E). Overall, these data suggest that ASXL1 may be a downstream target of SOX2.

3.2. The ASXL1 promoter responds to SOX2

To determine whether ASXL1 is a direct target of SOX2, we first isolated a 5.9 kb fragment of the human *ASXL1* promoter from genomic DNA from HEK293 cells and subcloned it into the pGL3 basic luciferase reporter plasmid. Subsequently, cell extracts were obtained from HEK293 cells transfected with the *ASXL1* promoter-driven luciferase reporter and a SOX2 expression vector, and were subjected to luciferase assays. As shown in Fig. 2A, reporter gene activity driven by the *ASXL1* promoter was 20-fold higher than that produced by the control plasmid. *ASXL1* promoter activity was increased sixfold by overexpression of SOX2. To map the SOX2-responsive region in the *ASXL1* promoter, we generated a series of deletions in the *ASXL1* promoter and performed luciferase assays in the absence and presence of SOX2 (Fig. 2B). The effect of SOX2 on luciferase activity was observed with a region of the *ASXL1*

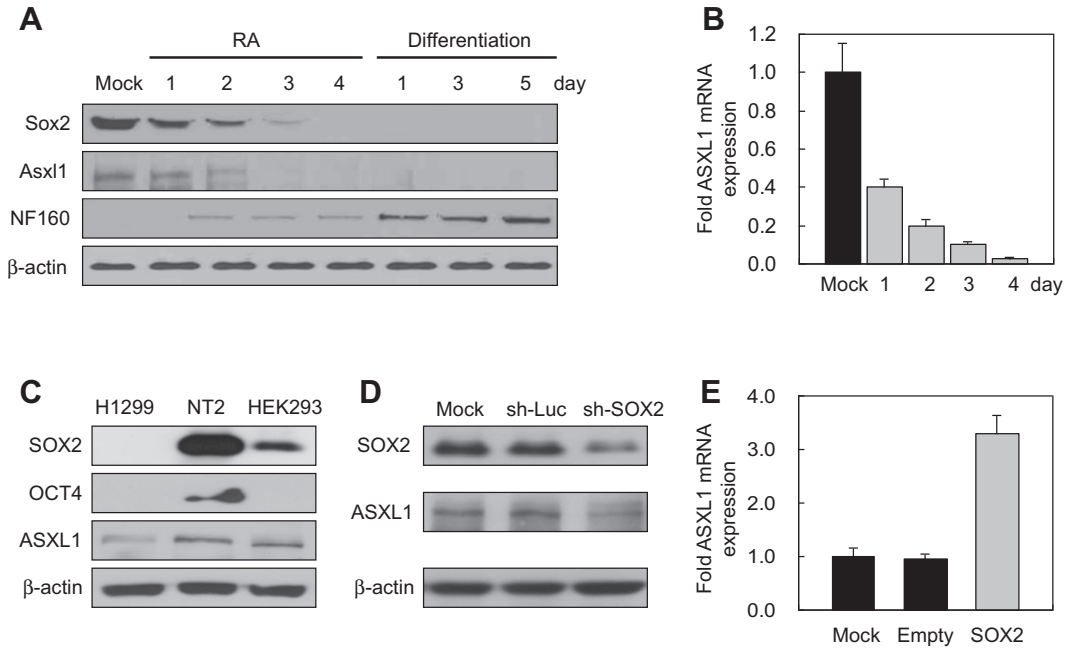

Fig. 1. Correlation between SOX2 and ASXL1 expression. (A) Downregulation of endogenous Sox2 and Asxl1 during RA-induced differentiation of P19 murine teratocarcinoma cells. RA (1 μM) was administered during EB formation in bacterial Petri dishes. EBs were differentiated in culture dishes in the absence of RA. Protein expression was monitored by western blotting (WB). NF160 and β-actin were used as a neural marker and loading control, respectively. (B) Downregulation of *Asxl1* mRNA expression during EB formation in the presence of RA. The mRNA level was determined by RT and real-time PCR (qPCR). (C) Expression of endogenous SOX2 and ASXL1 in human NT2, HEK293, and H1299 cells. (D) Effect of SOX2 knockdown on the level of ASXL1 in NT2 cells. SOX2 was depleted in SOX2-rich NT2 cells using an sh-knockdown system. (E) Effect of SOX2 overexpression on the expression of *ASXL1* mRNA in SOX2-deficient H1299 cells. A SOX2 expression vector was introduced into H1299 cells. At 24 h post-transfection, total RNA was isolated and the mRNA level was determined by RT-qPCR.

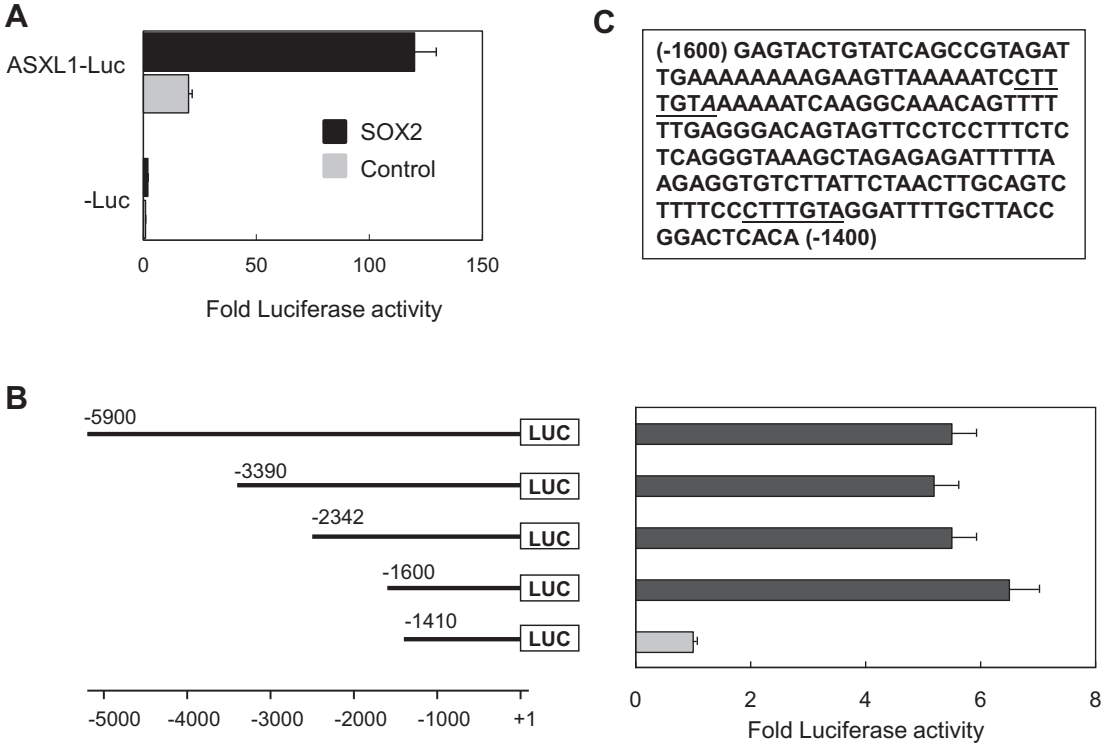

Fig. 2. Identification of SOX2 binding sites in the *ASXL1* promoter. (A) Effect of SOX2 on *ASXL1* promoter activity. A 5.9-kb fragment of the human *ASXL1* promoter, obtained from HEK293 cells, was subcloned into a luciferase expressing vector. The resulting *ASXL1*-luciferase reporter was used in luciferase assays in the absence and presence of SOX2 overexpression. (B) Identification of an SOX2-responsive region in the *ASXL1* promoter. An *ASXL1* promoter deletion series was generated by PCR. SOX2 responsiveness was determined by luciferase assay. Data represent the mean ± standard deviation of three independent experiments. (C) Location of the SOX2 binding sites. The sequence between −1600 and −1400 bp is shown. Known consensus SOX2 binding sites are underlined.

promoter spanning −5900 to −1600 bp. However, no SOX2 effect was examined when the promoter region upstream of −1410 bp was deleted, suggesting that the SOX2 response region in the *ASXL1* promoter lies between −1600 and −1410 bp. Through computer-aided analysis of this region, we found two putative SOX2 binding sites (two CTTTGTA sequences; known SOX2 binding sequence is WACAAWG, where *W* = *A*/*T*) [22] (Fig. 2C). These sites were conserved in the mouse Asxl1 promoter (data not shown), suggesting that SOX2-mediated ASXL1 promoter activation occurs in both human NT2 cells and murine P19 embryonic cells.

3.3. SOX2 binds to the identified sites in the ASXL1 promoter

To determine whether SOX2 binds to the identified binding sites in the ASXL1 promoter, we first mutated these sites and analyzed SOX2-responsive activation of the mutated promoter by luciferase assay (Fig. 3A). Compared to the wild-type *ASXL1* reporter, the promoter activities of single mutants in response to SOX2 were decreased by about 40%. Double mutation reduced promoter activity to background levels, suggesting that both SOX2 binding sites are required for SOX2-induced activation of the *ASXL1* promoter. Next, to determine whether SOX2 is capable of binding to the *ASXL1* promoter *in vivo*, we performed ChIP analysis. The primer sets used in this ChIP analysis are shown in Fig. 3B. ChIP assays were performed using H1299 and NT2 cells with and without exogenous overexpression of SOX2. As shown in Fig. 3C, a clear PCR product covering the two SOX2 binding sites was generated with the SOX2 antibody in both H1299 and NT2 cells, whereas no PCR product was observed when primers within exon 2 of *ASXL1* were used as a control. These results were further quantitated by real-time PCR (Fig. 3D). Both exogenous and endogenous SOX2 preferentially bound to the ASXL1 promoter with a difference in binding affinity, which may be ascribed to differential expression of SOX2. No enrichment of SOX2 was detected when exon 2 was used as the PCR template (data not shown). Taken together, our findings strongly suggest that SOX2 upregulates *ASXL1* gene transcription via direct binding to the SOX2 response element in the *ASXL1* promoter. These results suggest the existence of a functional link between SOX2 and ASXL1 in undifferentiated ES cells.

3.4. ASXL1 knockdown mimics SOX2 depletion in EB formation

To address the biological significance of the regulation of *ASXL1* expression by SOX2, we investigated the role of ASXL1 in maintaining self-renewal activity in ES cells. To this end, we used murine E14TG2a cells and generated stable cells with either Sox2 or Asxl1 depletion using an sh-knockdown system. As expected, Asxl1 protein expression was significantly reduced in Sox2-depleted stable cells (Fig. 4A). Surprisingly, Asxl1 knockdown reduced the expression of Sox2 and Oct3/4 without affecting the level of Nanog, another pluripotency factor (Fig. 4B). These results suggest that interplay between Asxl1, Sox2, and Oct3/4 modulates pluripotency in ES cells, while the core pluripotency factors Sox2, Oct3/4, and Nanog form a complex circuit by regulating each other's expression [19]. Finally, the role of Asxl1 in ES cells was investigated by monitoring its effect on the formation of EBs, a characteristic of ES cells. When compared to control sh-luciferase cells, the EB size for Asxl1-depleted ES cells was greatly reduced, similar to Sox2-knockdown cells (Fig. 4C). The average size of 50 EBs with diameters >10 μm was unchanged in both Asxl1- and Sox2-depleted cells during 3 days of EB formation, while the EB size in control cells gradually increased (Fig. 4D). Overall, our findings indicate that the Sox2 target gene *ASXL1* may play a crucial role in maintaining the pluripotency of ES cells.

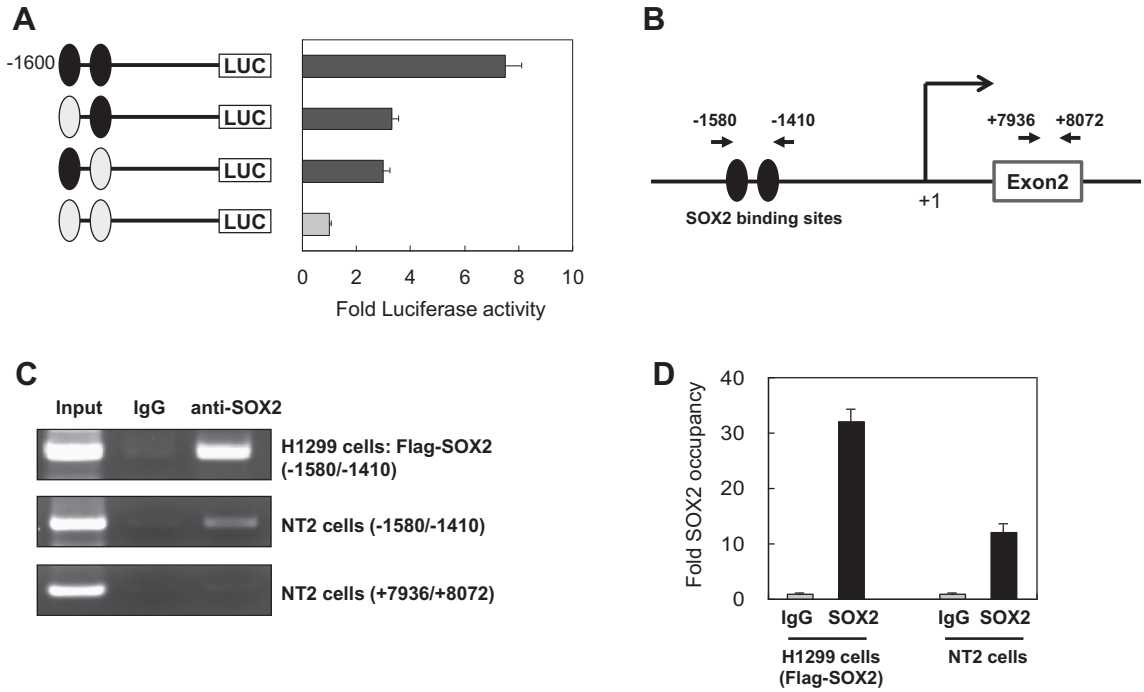

Fig. 3. Binding of SOX2 to the ASXL1 promoter *in vivo*. (A) Role of identified SOX2 binding elements in the response to SOX2. The core sequence (CTTG) of the consensus SOX2 binding site was mutated to TCGA at both sites. The resulting mutant reporters were subsequently used in luciferase assays in the absence and presence of SOX2 overexpression. Data represent the mean ± standard deviation (SD) of three independent experiments. (B) Schematic view of the SOX2 binding elements in the *ASXL1* gene. Primer sets for ChIP analysis were found in the upstream and exon 2 of the *ASXL1* gene. (C) Binding of SOX2 to the *ASXL1* promoter *in vivo*. ChIP assay (IP with anti-SOX2 antibody followed by PCR with specific primer sets) was performed using H1299 cells overexpressing Flag-SOX2 as a result of transient transfection and untreated NT2 cells. The final PCR products were analyzed by conventional PCR. IgG was used as a negative control. (D) Quantitation of the binding of SOX2 to the *ASXL1* promoter. ChIP was performed as described above and the final PCR products were analyzed by real-time PCR. Fold SOX2 occupancy represents SOX2 binding compared to the IgG control. Data represent the mean ± SD of three independent experiments.

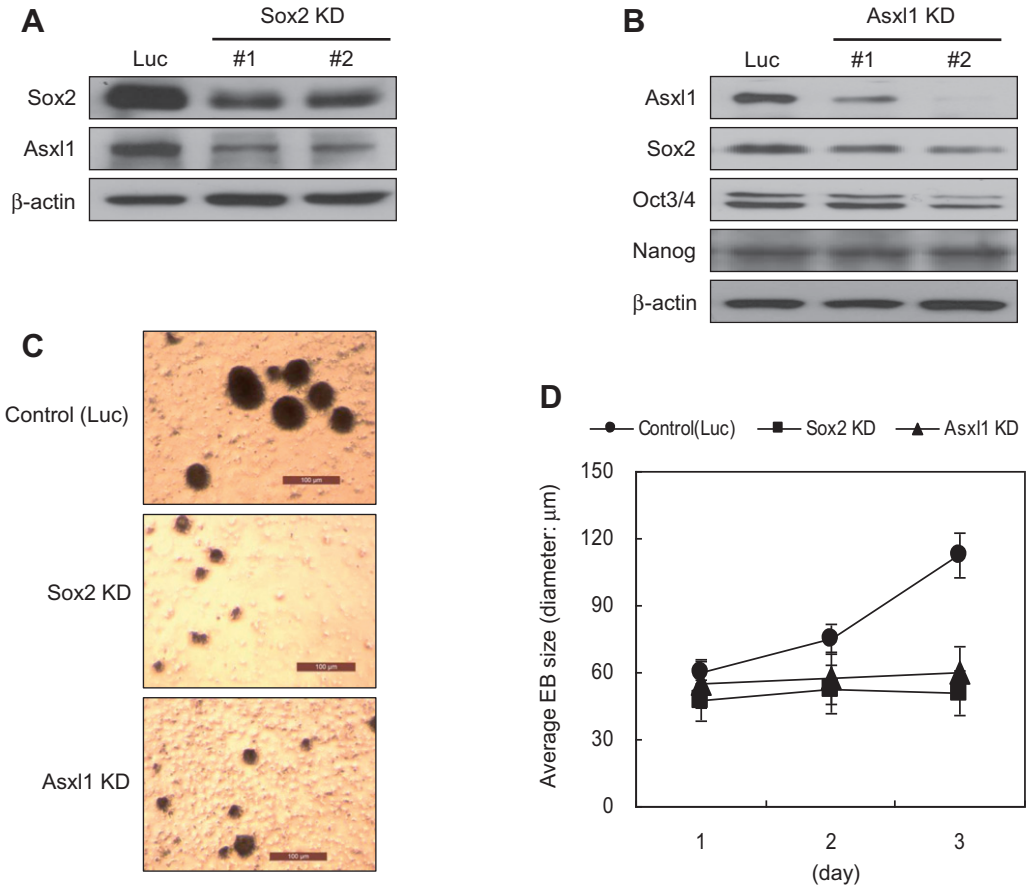

Fig. 4. Effect of ASXL1 depletion on EB formation. (A) Knockdown of endogenous Sox2. Sox2 knockdown (Sox2 KD) was confirmed by WB analysis of murine E14TG2a cells stably transfected with 2 μg sh-Sox2. Two stable cell lines were selected and are shown; sh-luciferase (Luc) was used as a negative control. (B) Effect of Asxl1 depletion on the expression of core pluripotency factors. Two stable cell lines in which Asxl1 was knocked down were selected for WB analysis. (C) Effect of Sox2 or Asxl1 knockdown on EB formation. Two depleted cell lines and control cells were grown in bacterial Petri dishes in the absence of LIF for 3 days. EBs were visualized at 200× magnification using a Leica microscope (scale bar, 100 μm). (D) Quantitation of the effects of knockdown. The sizes (diameters) of 50 EBs with diameters >20 μm were averaged using Leica LAS AF Lite software. Changes in average EB size were monitored at three different time points. Data represent the mean ± standard deviation of three independent experiments.

In summary, we found that SOX2 positively regulates expression of the *ASXL1* gene. To support this finding, we isolated the enhancer and promoter regions of the *ASXL1* gene and identified two SOX2 binding sites. We subsequently demonstrated that these sites are required for SOX2 binding and activation *in vivo*. Finally, the biological relevance of SOX2-activated *ASXL1* gene expression was investigated in murine ES cells. Asxl1 depletion resulted in reduced expression of the pluripotency factors Sox2 and Oct3/4 and reduced the sizes of EBs. In addition, alkaline phosphatase staining, another feature of ES cells, was weaker in Asxl1-depleted ES cells (data not shown). We also monitored the expression of lineage-specific genes. The ectoderm markers *Snail1* and *Fgf5* and the mesoderm marker *Brachyury* were upregulated in both Asxl1- and Sox2-depleted cells, while the endoderm marker *Gata4* only responded to Sox2 knockdown, and the trophectoderm marker *Eomes* remained unchanged in both cell types (data not shown). These results suggest that Sox2 and Asxl1 may share a common pathway, but in some cases play distinct roles in regulating the pluripotency of murine ES cells. Overall, we speculate that ASXL1, by forming a novel regulatory circuit with SOX2, may provide additional complexity in maintaining the pluripotency and differentiation of ES cells.

Acknowledgments

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