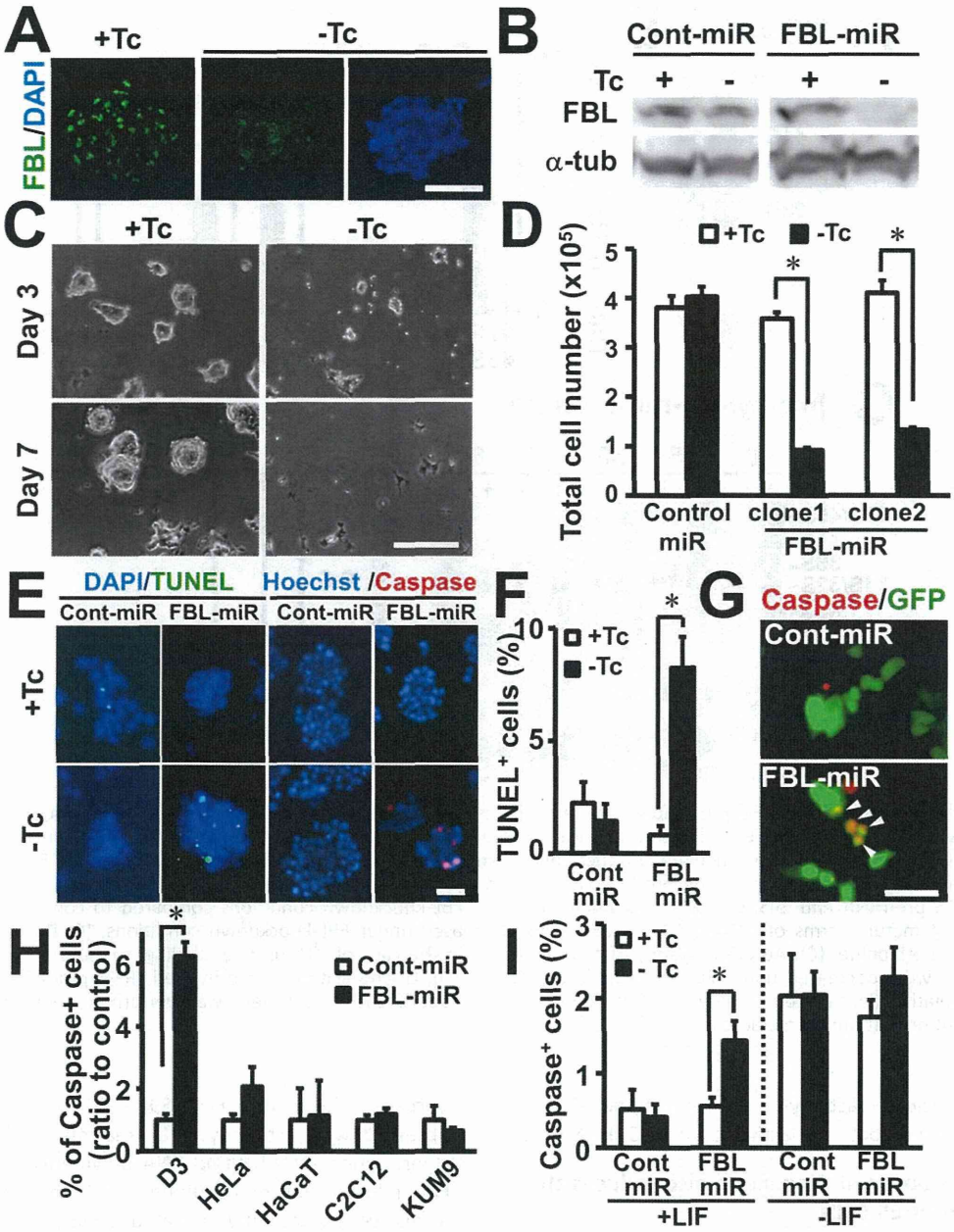

Figure 3. FBL is indispensable for the survival of embryonic stem (ES) cells. Tc-inducible FBL miRNA-expressing mouse ES cell lines were established and analyzed. **(A):** Immunofluorescence analysis of FBL expression. Upon withdrawal of Tc, FBL expression was notably decreased (middle and right). Cells were immunostained after 2 days of culture in the absence of Tc. **(B):** Western blot analysis of FBL expression in (A). A control miRNA did not affect the expression of FBL. **(C):** Morphological changes in FBL-knockdown ES cells under feeder-free culture conditions. After induction of FBL miRNA (−Tc), colonies of ES cells decreased in size and number at day 3 and had gradually disappeared by day 7. **(D):** Quantification of (C) by counting cell numbers after 3 days of culture. The data for control miRNA were shown on the left. **(E):** Significant increase in the number of TUNEL- or activated caspase-positive cells among FBL-knockdown ES cells cultured for 2 days in the absence of Tc. **(F):** Quantification of TUNEL-positive cells in (E). **(G):** Activated caspase staining of ES cells. GFP expression indicates the transfected cells with either GFP-control miRNA or GFP-FBL miRNA expression vector. **(H):** Apoptotic cell death caused by FBL-knockdown was significant in ES cells but not in other cell lines. Quantification of activated caspase-positive cells was performed 2 days after transfection with GFP-control miRNA or GFP-FBL miRNA expression vector. **(I):** Quantification of caspase-positive cells in ES cells. In the presence of LIF, ES cells were cultured with or without Tc for 2 days. In the absence of LIF, ES cells were precultured in the presence of Tc for 7 days, further cultured with or without Tc for 2 days, and percentage of caspase-positive cells was analyzed. *, $p < .01$ in (D, F, and H). *, $p < .05$ in (I). Scale bars = 30 μm (A), 300 μm (C), and 50 μm (E, G). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FBL, fibrillarin; GFP, green fluorescent protein; LIF, leukemia inhibitory factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; Tc, tetracycline.

through the binding of ribosomal protein L11 with a p53 inhibitor, Mdm2 [30, 31]. A previous report suggested that upon induction of ribosomal stress, L11 is released from nucleoli into the nucleoplasm, where it associates with Mdm2; this, in turn, rescues p53 from the Mdm2-dependent p53 degradation pathway [31]. Our coimmunoprecipitation experiments also confirmed enhanced binding of Mdm2 with L11 in FBL-knockdown ES cells (Fig. 5D). These results indicate

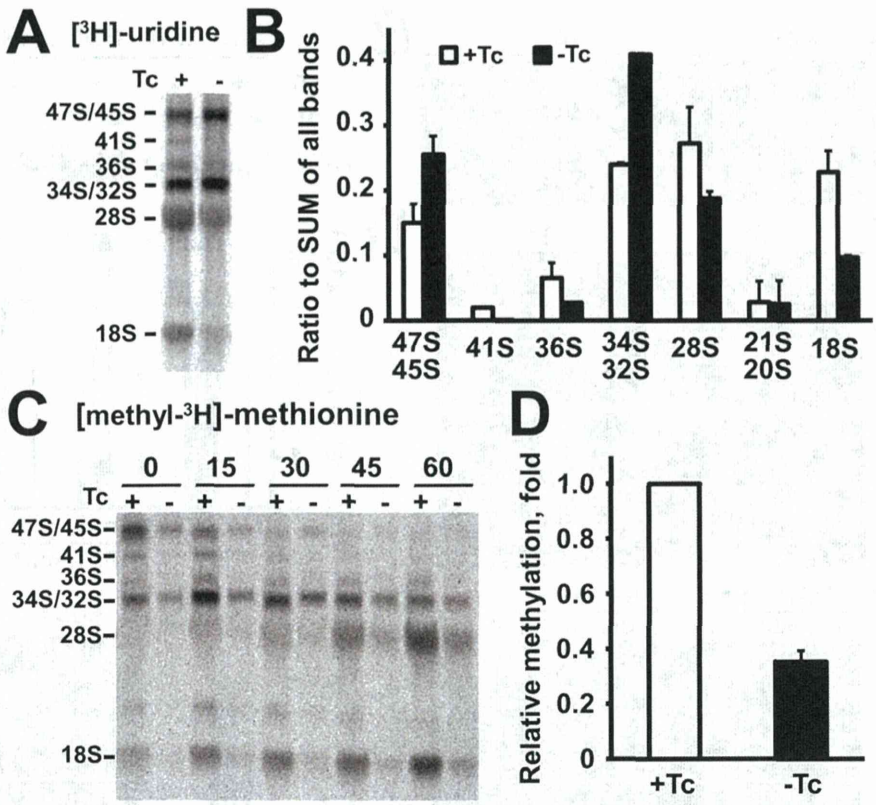

Figure 4. Knockdown of fibrillarin (FBL) in embryonic stem (ES) cells disturbs normal processing of pre-rRNA. **(A, B):** Pulse-chase labeling of ES cells with [^{3}H]-uridine. The ES cells were cultured with or without Tc for 2 days before labeling. (A): Autoradiography of total RNAs. The levels of 41S and 36S intermediate products and mature rRNA, 28S and 18S, were decreased in FBL-knockdown ES cells (−Tc). (B): Quantification of rRNA processing products. Each band was measured and divided by the sum of all products. Intermediate products, 47S/45S pre-rRNA and 34S/32S, were accumulated under FBL-knockdown conditions compared to control conditions. In contrast, the levels of mature forms of rRNA, 18S and 28S, were decreased under FBL-knockdown conditions. **(C, D):** Pulse-chase labeling with [methyl-^{3}H]-methionine. (C): Autoradiography of total RNAs. As in the case of [^{3}H]-uridine labeling, the levels of 41S and 36S intermediate products were decreased under FBL-knockdown conditions. (D): Quantification of methylated rRNA products. Graph shows the comparison of relative densitometric values of methylated rRNA products. Each experiment was performed two times, and the data were averaged. Abbreviation: Tc, tetracycline.

that impaired nucleolar activity by decreasing the rRNA production specifically induces the activation of p53 in ES cells.

Reduction of Ribosomal Biogenesis also Induces the Differentiation of ES Cells

Whereas complete knockdown of FBL led to apoptosis in ES cells (Fig. 3), partial knockdown of FBL promoted their differentiation. In ES cells cultured with 5 ng/mL Tc for 6 days, the FBL protein level decreased to one third of that measured under control conditions (Fig. 6B, 6C), which is similar to the level determined in differentiated cells cultured without LIF (Fig. 1B–1D). These cells showed a morphological transition of nucleoli, that is, from large condensed to small scattered foci, and flattened cellular morphology, even in the presence of LIF (Fig. 6A, 6B). qRT-PCR analysis revealed a fivefold increase in the primitive ectodermal marker *Fgf5* and a more than twofold increase in the mesodermal markers *Flk1* and *Brachyury* (*T*). In contrast, pluripotent markers were not affected except for *Oct4* and *Rex1*, which were slightly upregulated (Fig. 6D). In addition, the neural progenitor markers *Pax6* and *Nestin* were upregulated by about twofold compared to the controls (Fig. 6D). Immunofluorescence staining also confirmed the upregulation of these differentiation-specific markers (Supporting Information Fig. S9). Similar results were also observed with actinomycin D treatment, which blocks rRNA transcription by inhibiting RNA polymerase I (Fig. 6E). Under FBL-reduced culture conditions, p53 and its phosphorylated forms were transiently activated at around day 2 to day 3 of culture (Supporting Information Fig. S10). As shown in Figure 6F, 6G, FBL-knockdown-dependent induction of the early differentiation markers *T* and *Fgf5* was strongly inhibited by the p53-specific inhibitor pifithrin-α (PFTα), which indicates that the FBL-p53 axis controls the differentiation of ES cells. The activation of p53 was also observed during spontaneous differentiation of ES cells cultured in the absence of LIF. Stable expression of FBL inhibited p53 activation and differentiation (Supporting Information Fig. S11). One of the kinases responsible for the phosphorylation of p53 at Ser389 is casein kinase II (CK II) [32]. Interestingly, the expression of CK II was induced concomitantly with S389 phosphorylation of p53 (Supporting Information Fig. S11A). We further examined the specificity of p53 phosphorylation by CK II with a CK II-specific kinase inhibitor, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole. However, this inhibitor did not show a clear inhibition of p53 phosphorylation (data not shown), suggesting the existence of other kinases that can phosphorylate p53 in ES cells.

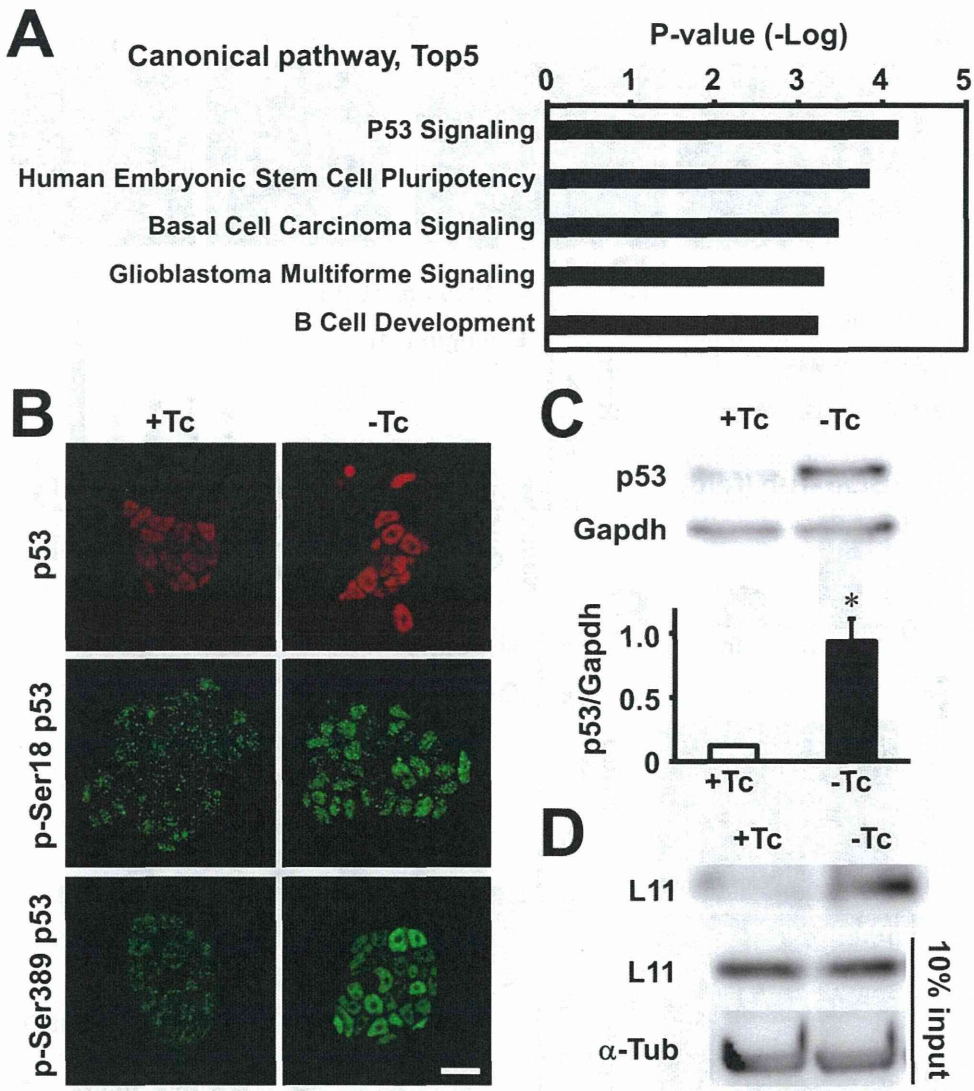

Figure 5. p53 signaling was specifically activated in fibrillarin (FBL)-knockdown embryonic stem (ES) cells. **(A):** Pathway analysis of microarray data obtained with FBL-knockdown ES cells. ES cells were cultured in the absence of Tc for 2 days. p53 signaling was identified as the most activated canonical pathway after knockdown of FBL. **(B):** Immunofluorescence analysis of p53 and p53 phosphorylation at Ser18 and Ser389 in FBL-knockdown ES cells cultured in the absence of Tc for 2 days. **(C):** Western blot analysis of p53 protein in ES cells cultured for 2 days without Tc. The lower graph shows the comparison of relative densitometric values of the bands. Values were normalized to Gapdh. Expression levels of p53 were 7.8-fold upregulated in the absence of Tc (FBL-knockdown conditions). **(D):** Coimmunoprecipitation analysis of L11 and Mdm2 interaction using ES cells cultured for 2 days after knockdown of FBL. Scale bar = 20 μm (B). *, $p < .01$ in (C). Abbreviation: Tc, tetracycline.

Next, we asked whether the activity of rRNA production in nucleoli could regulate neuronal differentiation of ES cells. Using Tc-off miRNA FBL knock-in ES cells, monolayer neural differentiation was performed according to a previously published report [33]. After 7 days of culture with 5 ng/mL Tc, the differentiation of $Tuj1^+$ neural cells was enhanced (9.0% ± 1.5%) compared to those cultured under control conditions (1,000 ng/mL Tc) (3.1% ± 0.5%) (Fig. 7A). Embryoid body-based differentiation also revealed a similar increase in neural differentiation in FBL-partial knockdown cells (Fig. 7B). The numbers of both $Tuj1^+$ and $NF200^+$ cells were increased as the Tc concentration decreased (Fig. 7C, D). In contrast, overexpression of FBL showed a rather inhibitory effect on this differentiation (Fig. 7E, 7F). Tc-off-induced FBL knockdown-dependent differentiation was completely inhibited by PFTα, but not by the caspase-specific inhibitors Z-VAD-FMK and Z-VDVAD-FMK (Fig. 7G, 7H), indicating that a reduction in FBL levels induces the differentiation of ES cells in a p53-dependent manner, which is independent from apoptosis-dependent activation of p53. These results demonstrate that the decrease in rRNA production can control the efficiency of ES cell differentiation.

Discussion

Nucleoli are the centers of rRNA biosynthesis, and their size reflects ribosomal activity, cell-cycle progression, and proliferation [6, 34]. Hypertrophy of the nucleoli has been reported as one of the most distinctive cytological features of cancer cells [35]. In contrast, defects in ribosomal activity are associated with several inherited bone marrow failure syndromes, for

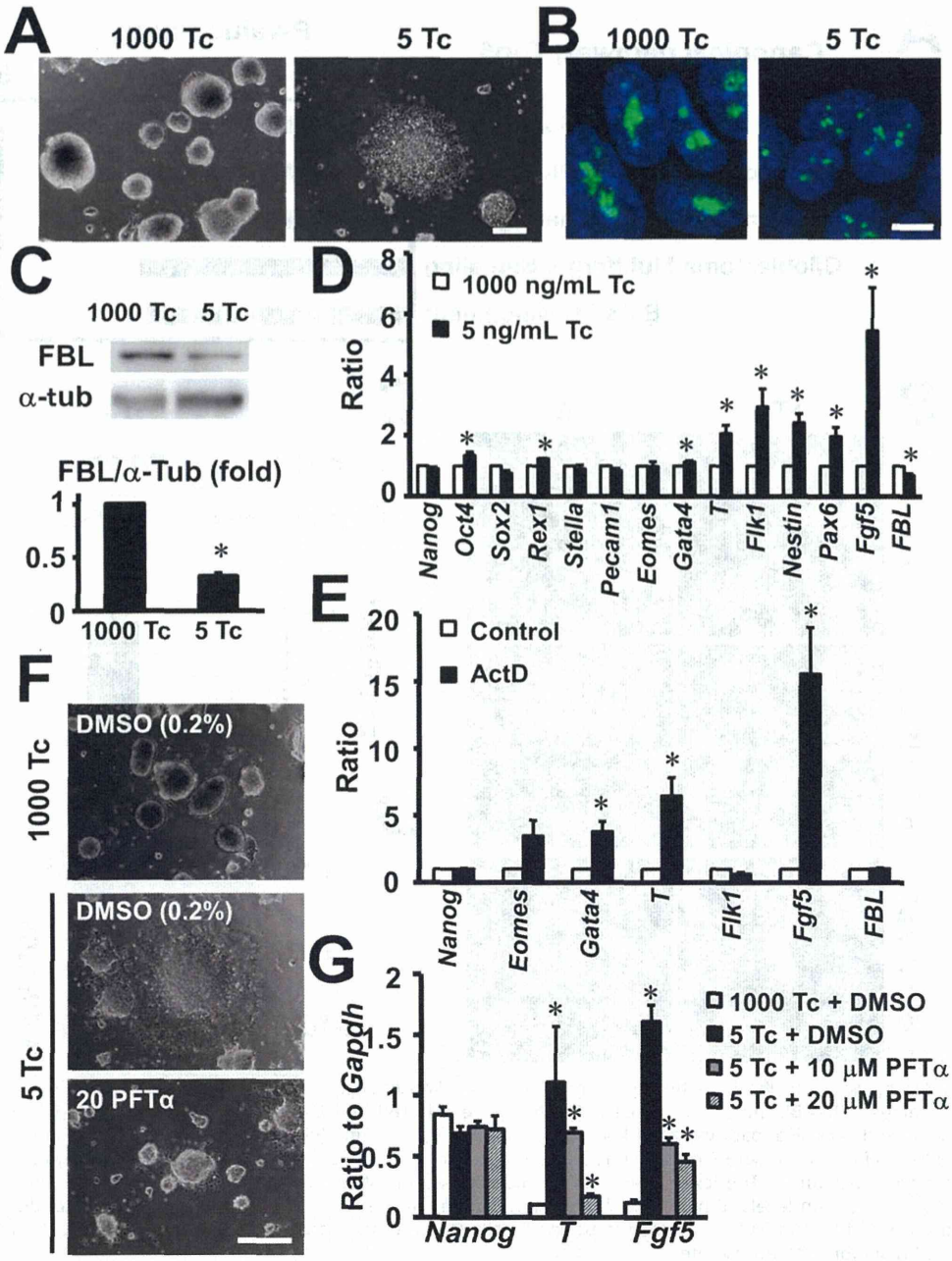

Figure 6. Reduction of FBL expression induces differentiation marker expression via the p53 signaling pathway under self-renewal culture conditions. **(A):** Embryonic stem (ES) cells cultured for 6 days with 5 ng/mL of Tc resulted in the loss of the densely packed morphology. **(B):** Immunofluorescence analysis of FBL in (A). **(C):** Western blot analysis of ES cell extracts after partial knockdown of FBL. FBL expression was decreased to about one-third of that of control conditions after 6 days of culture with 5 ng/mL Tc. **(D):** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of differentiation- and pluripotency-specific marker expression for each culture condition. After 6 days of culture, the expression of differentiation markers increased, whereas the expression of pluripotency markers was not changed under FBL-reduced conditions. **(E):** qRT-PCR analysis of differentiation- and pluripotency-specific marker expression 24 hours after administration of 4 ng/mL ActD. **(F):** Morphological changes in ES cells. ES cells were cultured under self-renewal conditions, and PFTα or DMSO was added from the beginning of culture. In the presence of PFTα, colonies retained their densely packed morphology, even after partial knockdown of FBL. **(G):** qRT-PCR analysis of the expression of differentiation markers. PFTα treatment suppressed the expression of differentiation markers, *T* and *Fgf5*, in a dose-dependent manner. *, $p < .01$ in (C, E, and G); *, $p < .05$ in (D). Scale bars = 300 μm (A), 10 μm (B), and 500 μm (F). Abbreviations: ActD, actinomycin-D; DMSO, dimethylsulfoxide; FBL, fibrillarin; PFTα, pifithrin-α; Tc, tetracycline.

example, Diamond-Blackfan, and characterized by a decreased number of erythroid progenitors as well as a variety of abnormalities during development, for example, congenital and growth retardation [36, 37].

In ES cells, large and condensed nucleoli are one of the hallmarks of pluripotency [4]. The morphology of the nucleoli changes from large to scattered, small foci during the differentiation of ES cells. In this study, we have shown that ES cells

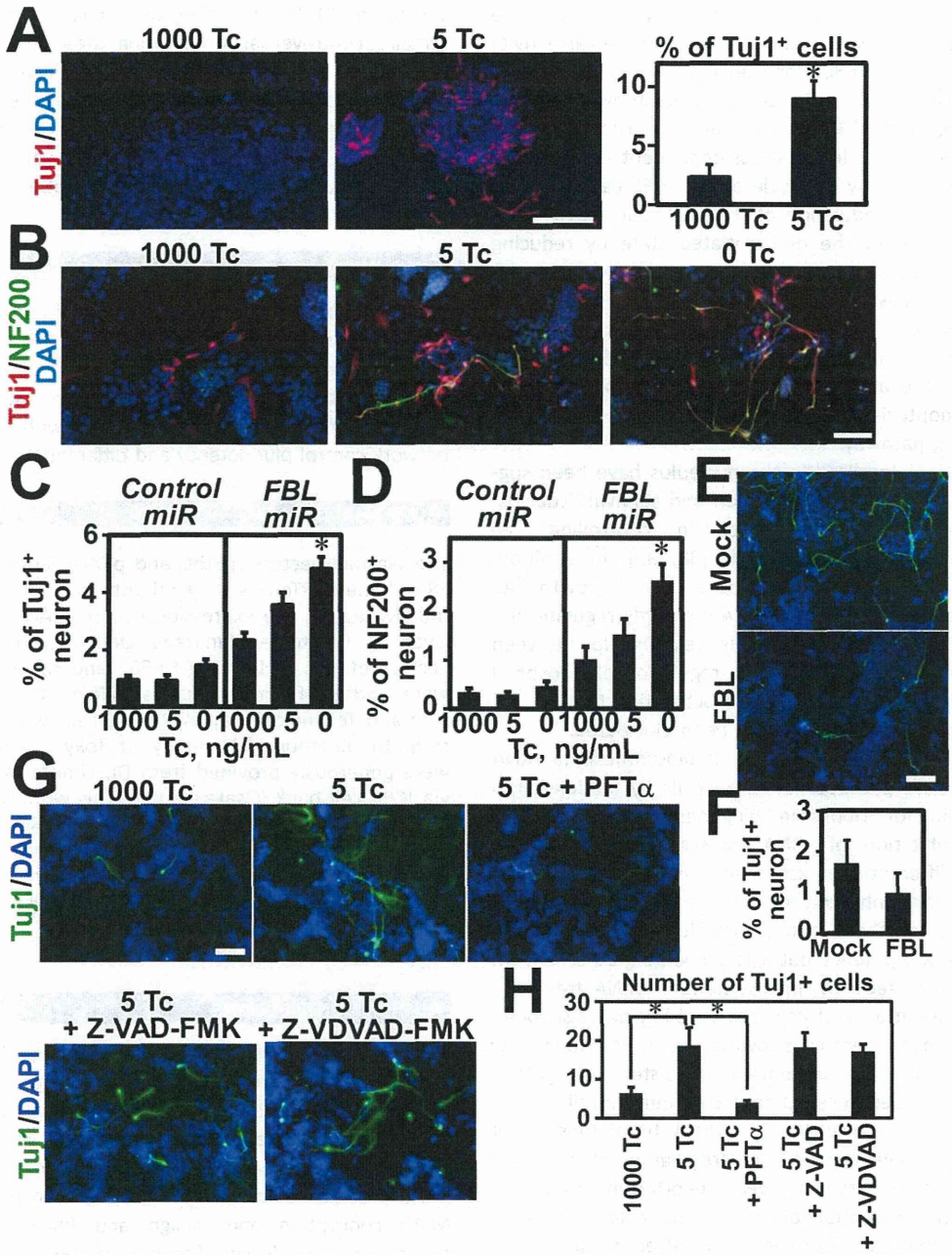

Figure 7. Partial knockdown of FBL promoted neural differentiation of embryonic stem (ES) cells in a p53-depndent manner. **(A):** Neural differentiation of ES cells in monolayer culture. Cells were dissociated, attached to poly-L-lysine/laminin/fibronectin (PLL/LN/FN)-coated dishes, and cultured for 7 days. The concentration of Tc was changed to the indicated concentration (ng/mL) from the beginning of differentiation. (Left panels) Tuj1$^+$ cells were significantly increased only under FBL-reduced conditions. The graph shows the percentage of Tuj1$^+$ neurons for each culture condition. **(B):** Neural differentiation of ES cells by embryoid body-based culture. Embryoid bodies were cultured in a low cell attachment culture dish for 5 days, dissociated, attached to PLL/LN/FN-coated dishes, and cultured for 4 days. The concentration of Tc was changed on day 3 to the indicated concentration. **(C):** The percentage of Tuj1$^+$ neurons. The ES cells were differentiated as in (B) under the indicated culture conditions. **(D):** The percentage of NF200$^+$ neurons. The ES cells were differentiated as in (B) under the indicated culture conditions. **(E, F):** Overexpression of FBL slightly inhibited neural differentiation. (E) Neural differentiation of Tc-inducible FBL-expressing ES cells under monolayer culture. ES cells were cultured for 6 days as described in (A). (F) The percentage of Tuj1$^+$ neurons in (E) was counted. **(G, H):** Inhibition of p53 suppressed neural differentiation of ES cells. (G): Cells were cultured as in (A) with or without p53 and apoptosis inhibitors. All of the inhibitors were added from the beginning of differentiation. (H): The number of Tuj1$^+$ neurons in (G) was counted. *, $p < .01$ in (A, C, D, and H). Scale bars = 200 μm (A); 100 μm (B, G, H). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FBL, fibrillarin; PFTα, pifithrin-α; Tc, tetracycline.

stably expressing FBL, a critical regulator of rRNA synthesis, retained pluripotency-state-specific large nucleoli for a longer time in the absence of LIF and prolonged their pluripotency. In contrast, when the status of nucleoli was changed to the differentiated state by reducing the expression of FBL to the level of differentiated cells, the ES cells became partially differentiated even under self-renewing culture conditions. These results clearly indicate that the nucleolar state, that is, the activity of

ribosome biogenesis, controls the pluripotency of ES cells. We further demonstrated that a decrease in the nucleolar activity by partial knockdown of FBL promotes neural differentiation.

In FBL-knockdown ES cells, complete shutdown of rRNA biosynthesis triggered p53 activation and induced apoptosis. This could be due to nucleolar stress-dependent activation of p53 signaling followed by cell-cycle arrest in ES cells [30, 31]. On the other hand, modulation of the nucleolar activity to a level similar to that of the differentiated state by reducing FBL expression could mildly activate p53 and induce the differentiation of ES cells even in the presence of LIF. The differentiation of ES cells induced by the modification of rRNA biosynthesis was completely suppressed by a p53 inhibitor, PFTα. Therefore, the activity of rRNA biosynthesis could control not only apoptosis but also differentiation of ES cells via the p53 signaling pathway.

Several proteins localized in the nucleolus have been suggested to regulate ES cells proliferation and survival. Nucleostemin was shown to participate in controlling cell proliferation and survival in ES cells [38, 39]. In addition, nucleophosmin 1 is essential for mouse ES cell growth [40, 41]. However, these two proteins do not directly regulate biosynthesis of rRNA, and the curious relationship between unique nucleolar morphology, that is, regulation of ribosomal biogenesis and the ES cell-specific characteristics, that is, pluripotency and differentiation, has not been elucidated.

In this study, we demonstrated that biosynthesis of rRNA itself could regulate pluripotency of ES cells by modulating a principal protein for ribosome biogenesis, FBL, and also showed that inhibition of rRNA transcription triggers the expression of differentiation-associated markers by an RNA polymerase I-specific inhibitor, actinomycin D. Our results suggest that rRNA biosynthesis in the nucleolus is a novel regulator for not only pluripotency but also preventing ES cells from differentiating. Very recently, involvement of rRNA transcription in the proliferation and cell fate decision has also been reported in the other stem cells, ovarian germ line stem cells in *Drosophila* [42] and mouse hematopoietic stem cells [43].

For several decades, researchers have been describing the relationship between nucleolar size, rRNA biosynthesis, cell proliferation, and development. Downregulation of nucleolar activity during differentiation has been reported in various tissues. For example, reduction of the nucleolar size has been reported during maturation of the intestinal epithelium in rat [44], epidermal maturation in chick [45], and eye differentiation in *Drosophila* [46]. p53 is also expressed in various organs during development, and its expression level is significantly downregulated as differentiation proceeds [47, 48]. Although p53-null mice are viable, previous studies suggested the importance of p53 in normal development in several tissues, for example, the nervous system, eyes, hind limbs, and teeth [49, 50]. However, the regulatory mechanism of p53 remains controversial. Considering these in vivo observations, our results raise the hypothesis that downregulation of the ribosomal activity in nucleoli could trigger the activation of the p53 signaling pathway and induce differentiation of various tissues in vivo. Further study is needed to verify the relationship between nucleolar activity and differentiation in vivo.

Conclusion

We have identified a novel regulatory function of nucleoli in pluripotent stem cells. We have shown that biosynthesis of rRNA in nucleoli is a unique regulator of pluripotency as well as the differentiation of stem cells. Our study suggests that strict regulation of nucleolar activity together with the transcriptional network control pluripotency and differentiation of ES cells.

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Author Contributions

K.W.-S.: and H.T.: collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; K.E.: conception and design, collection and/or assembly of data, and data analysis and interpretation; K.M., H.I., and A.I.: collection and/or assembly of data; M.O. and M.N.: provision of study material; H.S.: conception and design; M.A.: conception and design and financial support; A.K.: conception and design, financial support, data analysis and interpretation, manuscript writing, and final approval of manuscript. K.W.-S., H.T., and K.E. contributed equally to this work.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interests.

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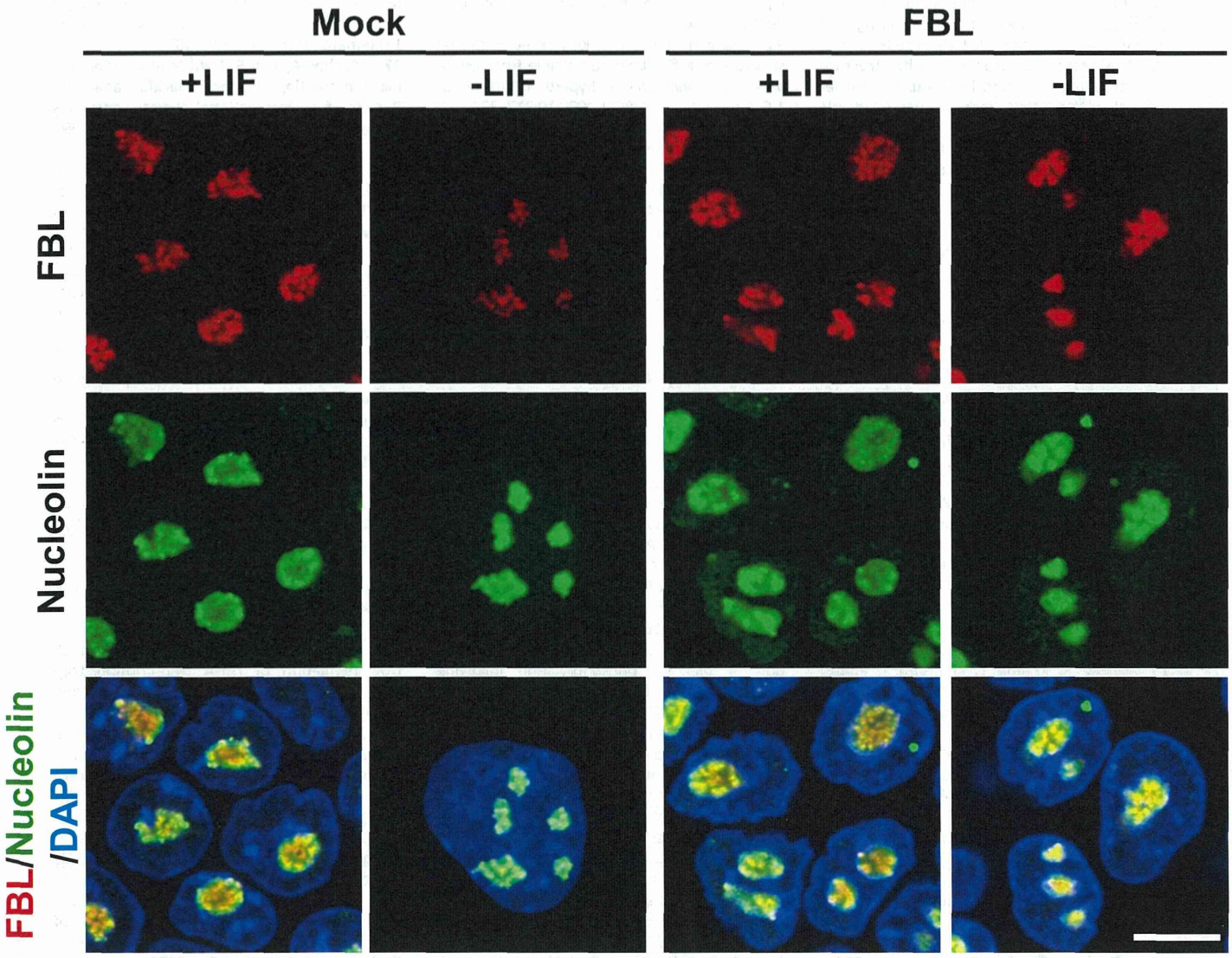

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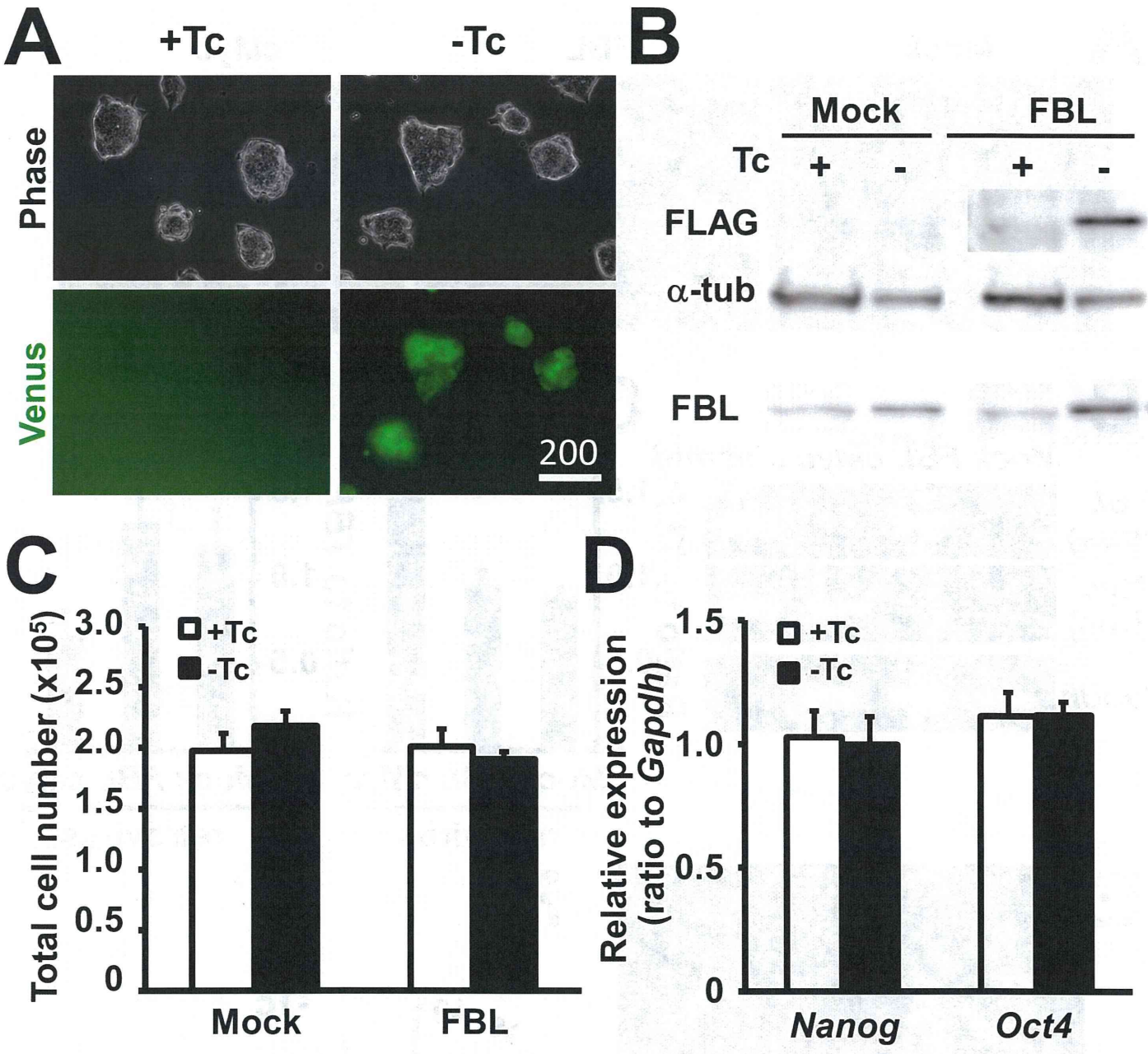

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