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UTF1 deficiency promotes retinoic acid-induced neuronal differentiation in P19 embryonal carcinoma cells

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ABSTRACT

UTF1 (undifferentiated embryonic cell transcription factor 1) is a marker for the pluripotency of embryonic stem cells. We found that UTF1-deficient clones, which were isolated from P19 embryonal carcinoma (EC) cells, showed higher neuron-differentiating potentials than the parental cell line. Consistent with this result, suppression of UTF1 expression in P19 cells by RNA interference enhanced retinoic acid (RA)-induced neuronal differentiation. Moreover, reconstitution of UTF1 expression in UTF1-deficient clones decreased their ability to undergo neuronal differentiation. Interestingly, the growth rates of UTF1-deficient P19 cells did not differ from that of parental cells in adherent cultures, but were increased in embryoid bodies during RA-induced differentiation. Furthermore, different from the parental cells, UTF1-deficient P19 clones could proceed to neuronal differentiation without forming embryoid bodies. Based on these results we proposed that endogenous UTF1 prevented P19 EC cells from neuronal differentiation, and that the loss of UTF1 directed EC cells toward the neuronal fate.

1. Introduction

Oct4, Sox2, and Nanog are transcription factors essential for maintaining the pluripotency of embryonic stem (ES) cells. At the top of the regulatory hierarchy, they establish a transcriptional circuit in human and mouse ES cells (Nishimoto et al., 2005; Rodda et al., 2005). Several of their downstream targets are the regulators controlling the differentiation process. UTF1 is originally identified in mouse EC and ES cells and is encoded by a target gene of Oct4 and Sox2 (Nishimoto et al., 1999; Okumura-Nakanishi et al., 2005). Forming a complex, Oct4 and Sox2 bind to the *UTF1* enhancer and synergistically induce UTF1 expression. Similar to Oct4, expression of UTF1 is rapidly decreased upon the differentiation of ES and EC cells (Trounson, 2006; Liu et al., 2010). In mouse ES cells, UTF1 maintains cell proliferation and promotes teratoma formation (Nishimoto et al., 2005). Suppression of UTF1 perturbs mesoderm and endoderm differentiation in mouse EC and ES cells (van den Boom et al., 2007). Experimental evidence shows UTF1 is a chromatin-associated protein that regulates specific epigenetic profiles required for differentiation (van den Boom et al., 2007; Kooistra et al., 2009, 2010). Also UTF1 improves the efficiency of human induced pluripotent stem cells generation from human fibroblast cells (Zhao et al., 2008). Taken together, UTF1 appears to be a key regulator for the pluripotency of ES cells.

Embryonic carcinoma (EC) cells, consider as malignant counterparts of ES cells, show pluripotent property as ES cells and are easily maintained in cell culture systems (van der Heyden and Defize, 2003). EC cells were derived from germ cell tumors. These tumors spontaneously occur in ovarian and testicular tissues and contain populations of undifferentiated stem cells. The P19 cell line, derived from a teratocarcinoma in C3H/HC mice, is one of the well-studied EC cells and manipulating it to multiple-lineage differentiation in culture system is feasible (Rossant and McBurney, 1982). Conventionally, RA induces P19 cells into neuronal and glial cell lineages while dimethyl sulfoxide (DMSO) induces them to form mesoderm derivatives including cardiac and skeletal muscle cells (McBurney et al., 1982; van der Heyden et al., 2003; Soprano et al., 2007). Isolated P19 clones have been shown to exhibit an increasing ability to differentiate into specific cell lineages in comparison to the parental cells (Habara-Ohkubo, 1996; Angello et al., 2006). In searching for cell models that are feasible for examining neuronal differentiation, we found that naturally occurred UTF1-deficient P19 clones are prone to RA-directed neuronal differentiation. Most interestingly, unlike parental P19 cells, UTF1-deficient P19 clones could undergo directed neuronal differentiation even without forming embryoid bodies (EBs). This study revealed a new direction for examining the functions of UTF1 in EC and ES cells.

Abbreviations: BrdU, 5′-bromo-doxy-uridine; DMSO, dimethyl sulfoxide; EBs, embryoid bodies; EC, embryonal carcinoma; ES, embryonic stem; IF, immunofluorescence; RA, retinoic acid; shRNA, small hairpin RNA; siRNA, small interference RNA; UTF1, undifferentiated embryonic cell transcription factor 1.

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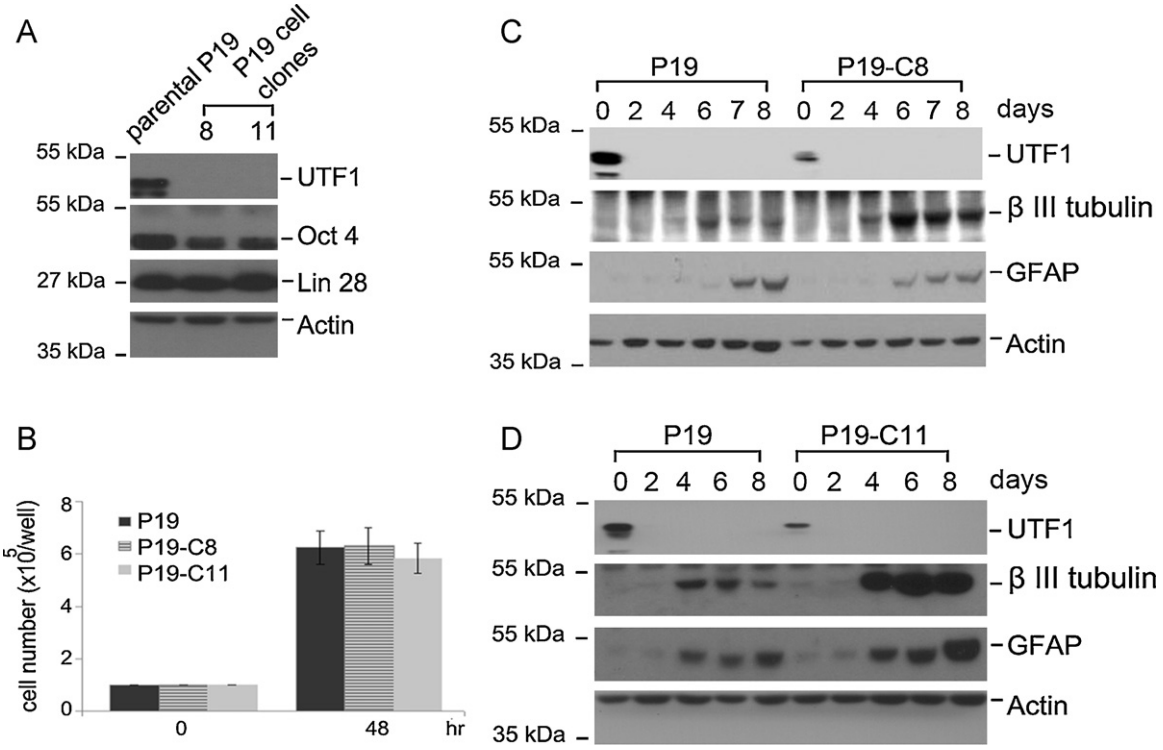

Fig. 1. UTF1-deficient P19 cells show normal proliferation and enhanced neuronal differentiation. (A) P19 and its derivative C8 and C11 cells were harvested for cell extract preparation. The expression levels of UTF1, Oct4, Lin28, and actin were examined by Western blot analyses using specific antibodies. (B) P19 variants were plated at a concentration of 1×10^5/well in a triplicated experiment. The numbers of cells were counted 48 h after plating to measure the cell proliferation. The data presented are the means and standard deviations of the measurements. (C and D) The procedure for neuronal differentiation was described in Section 2. Neuronal and glial differentiation was examined by Western blot analysis using anti-βIII tubulin and anti-GFAP antibodies. The expression of UTF1 was detected using an anti-UTF1 antibody. Actin expression was examined as the loading control.

2. Materials and methods

2.1. Cell cultures and transfection, cell differentiation, expression vectors, reagents, Western blotting, immune-fluorescence (IF) staining, and caspase assays

These experimental procedures, reagents, and three additional references (Bibel et al., 2004; Fu et al., 2008; Jones-Villeneuve et al., 1982) were described in Suppl. Materials and methods.

2.2. Hanging drop culture for EB formation

P19 and its derivative cells (1×10^5 cells/ml) were prepared in the presence of indicated chemicals. Drops (20 μl each) of cell suspension were placed on the lid of a 10-cm bacterial-grade Petri dish containing 10 ml PBS in the bottom to maintain the humidity. After 6 days, the images of EBs were documented using a microscopy and the size and morphology of EBs were calculated by the Photoshop CS4 program.

2.3. 5′-Bromo-doxy-uridine (BrdU) incorporation and caspase assay

BrdU incorporation was performed as described previously with modifications (Vaca et al., 2008). Three days into the P19 neuronal differentiation scheme, aggregated EBs were treated with 10 μM BrdU for 12 h. BrdU uptake was analyzed by immune-staining using a mouse anti-BrdU antibody (1:500; Sigma) followed by a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. The BrdU-labeled cells were measured by flow cytometry.

2.4. Axial elongation

The axial elongation assay was performed as previously described with modifications (Marikawa et al., 2009). P19 and its derivatives were suspended in the DMEM medium containing 2% bovine serum and 1.5% DMSO as described in the hanging drop culture method. At day 4 and day 6, cells were collected for Western blotting and images were documented using a microscopy. The Photoshop CS4 program was used to measure the circumference and area of individual aggregates in photographs. The elongation distortion index (EDI) was calculated as $\{(\text{circumference})^2/(\text{area}) \times 4\pi - 1\}$. The EDI presents the shape of an aggregate, but does not reflect the size of an aggregate. If the aggregate is completely spherical, EDI is zero; the more elongated the cell aggregates, the higher the EDI.

3. Results

3.1. UTF1-deficient P19 clones show enhanced RA-induced neuron differentiation

Aiming to find EC cells that were prone to neuronal differentiation, we used the limiting dilution method to isolate P19 clonal derivatives. Interestingly, few isolated clones (named P19-C8 and C11) showed low UTF-1 protein levels while other pluripotent markers, Oct4 and Lin28, were not affected as significantly (Fig. 1A). By IF staining, we found that P19-C8 and C11 cells had universal decreases of UTF1 expression (Suppl. Fig. 1). No differences in cell growth were observed between UTF1-deficient clones and parental P19 cells (Fig. 1B).

By morphological examinations, we found that P19-C8 and C11 clones showed more evident RA-induced neuronal

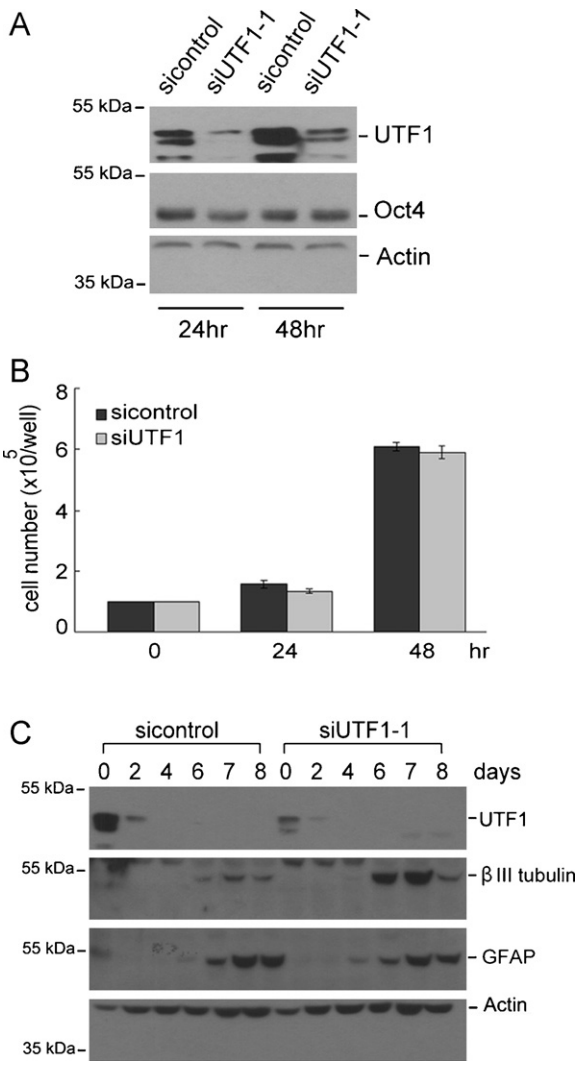

Fig. 2. Suppressing UTF1 expression promotes the neuronal differentiation of P19 cells. P19 cells were transiently transfected with a control (sicontrol) or UTF1 (siUTF1-1) siRNA and cultured for 36 h. (A) The protein levels of UTF1, Oct4, and actin in the transfected were determined by Western blotting using the indicated antibodies. (B) The transfected P19 cells were plated at a concentration of 1×10^5/well in a triplicated experiment. The numbers of cells were counted 24 and 48 h after plating to measure the cell proliferation. The data presented are the means and standard deviations of the measurements. (C) The transfected cells were subjected to neuronal differentiation induced by RA (0.1 μM). UTF1, βIII tubulin, GFAP, and actin expression in control and UTF1-knockdown cells was examined by Western blotting using indicated antibodies.

differentiation than did the parental cells. To confirm this observation, the expression levels of neuron-specific protein βIII tubulin and glia-specific protein GFAP were examined to assess neuronal differentiation. Upon RA-induced differentiation, both P19-C8 and C11 cells showed increases in βIII tubulin levels (Fig. 1C and D). However, the expression of GFAP in P19-C8 and C11 cells was not necessarily higher than that in P19 cells (Fig. 1C and D). Through IF staining, we also found that P19-C8 and C11 cells indeed contained more βIII tubulin-positive, differentiated neuronal cells upon RA treatment (Suppl. Fig. 2).

3.2. UTF1 suppression promotes RA-induced neuronal differentiation in P19 cells

P19-C8 and C11 clones might contain defects other than UTF1 deficiency. To confirm whether the lack of UTF1 is sufficient to promote neuronal differentiation, we used siRNA against UTF1

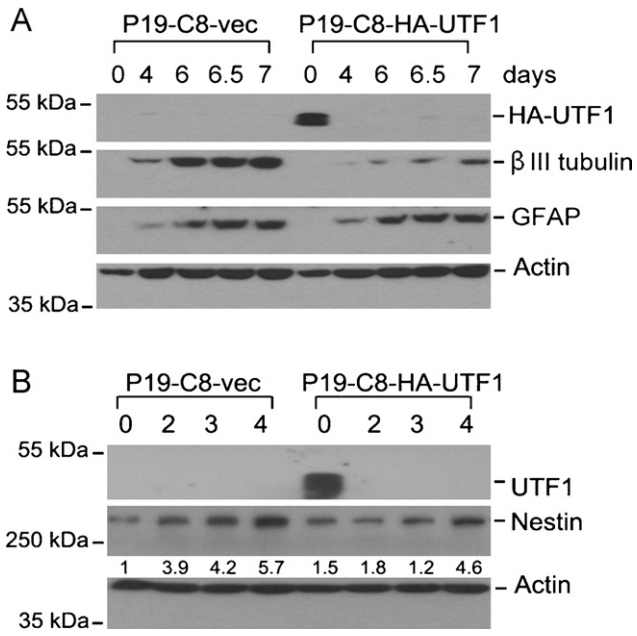

Fig. 3. Reconstitution of UTF1 expression in P19-C8 cells suppresses RA-induced neuronal differentiation. An empty vector or a HA-UTF1 vector was transiently transfected into P19-C8 cells. (A) After 36 h, transected cells were subjected to RA-induced differentiation and the cells were collected at the indicated time points. HA-UTF1 fusion protein was detected by an HA antibody. The expression levels of βIII tubulin and GFAP were examined by Western blotting. (B) The cells were collected between days 0 and 4. The expression of UTF1, nestin, and β-actin were examined by Western blotting using the respective antibodies. The quantified levels of nestin were normalized by the actin levels of the corresponding samples.

(siUTF1) to suppress UTF1 expression in P19 cells. Transient transfection of siUTF1-1 in P19 cells efficiently reduced the endogenous UTF1 level without affecting Oct4 protein expression (Fig. 2A). The cell growth of control and siUTF1-treated cells were not significantly different (Fig. 2B), indicating that UTF1 was not essential for maintaining P19 cell proliferation. This result was similar to what was observed in the P19-C8 and C11 cells.

Upon RA-induced differentiation, we found that the expression of βIII tubulin in UTF1-knockdown cells was higher than that in control cells (Fig. 2C), suggesting that suppression of UTF1 alone was sufficient to promote neuron differentiation. To avoid off-target effects, another UTF1 siRNA (siUTF1-2) was used to repeat the experiments. Consistently, siUTF1-2 efficiently decreased the endogenous UTF1 level and caused the cells to have higher βIII tubulin expression (Suppl. Fig. 3A). Also, knockdown of UTF1 expression by siRNA increased βIII tubulin-positive, differentiated neuron cells in RA-treated P19 cells (Suppl. Fig. 3B). Taken together, our data indicated that UTF1 deficiency did promote neuronal differentiation of P19 cells.

3.3. Reconstitution of UTF1 expression inhibits neuron differentiation in P19-C8 cells

To prove that the enhancement of neuron differentiation in P19 clonal cells was mainly due to UTF1 deficiency, we reconstituted UTF1 expression in them and examined its effect on RA-induced differentiation. IF staining showed that UTF1 transient expression universally increased the levels of this protein, which did not exceed those in parental cells, in P19-C8 cells (Suppl. Fig. 4). Upon RA treatment, lower expression of βIII tubulin was found in UTF1-transfected P19-C8 cells in comparison to that in vector-transfect cells (Fig. 3A). In contrast, no obvious difference was observed in GFAP protein expression between UTF1-transfected and control cells. The effect of UTF1 on βIII tubulin expression was evident only

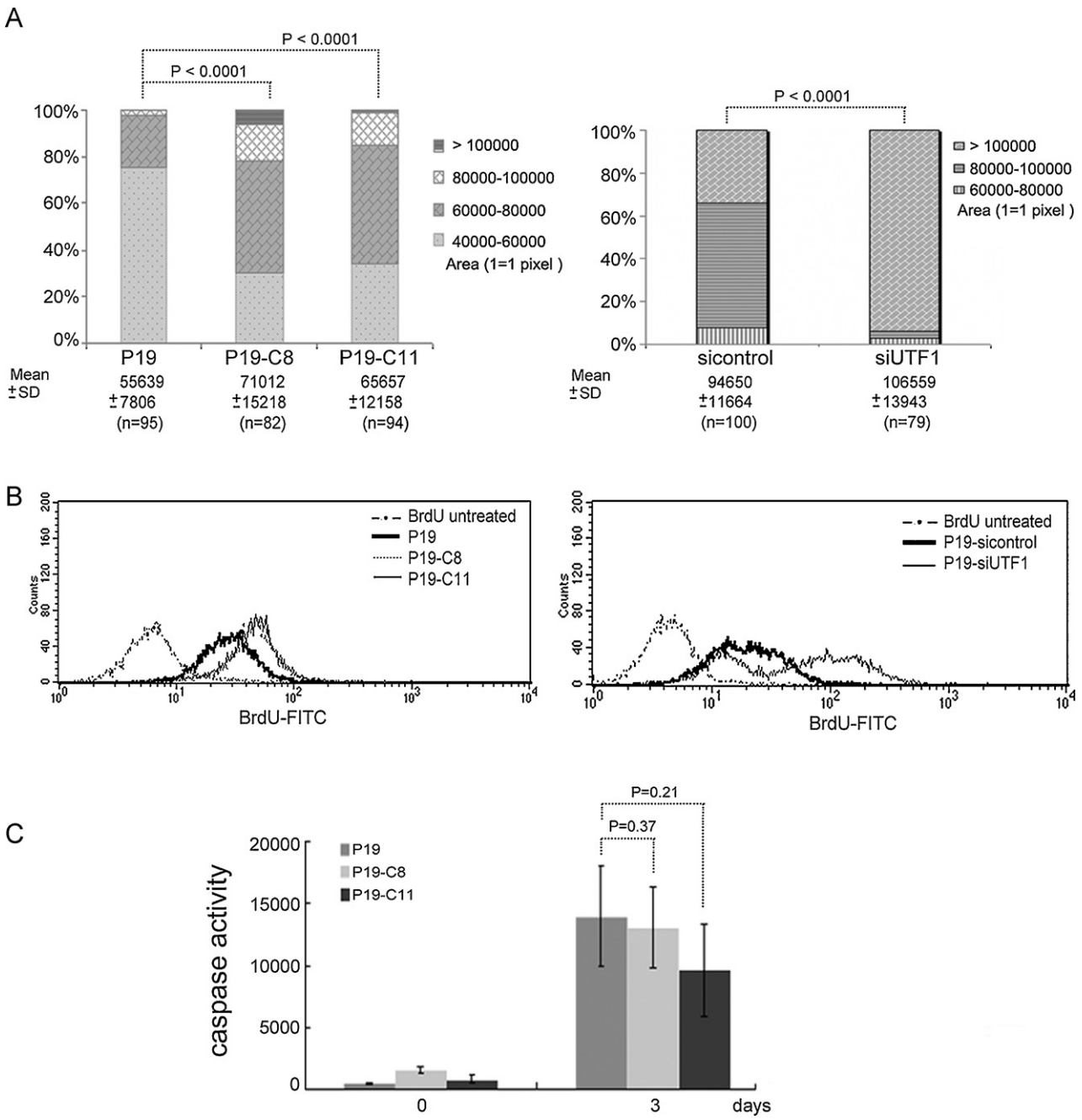

Fig. 4. Loss of UTF1 promotes P19 cell proliferation in embryoid bodies during RA-induced differentiation. (A) P19 cells (clones or siRNA-transfected cells) were aggregated in hanging drops containing 0.1 μM RA. After 6 days, the EBs were examined using a microscopy and the relative sizes of EBs were measured by the Photoshop CS4 program. The data were analyzed by unpaired, two-tailed Student's *t* test. The numbers of EBs analyzed in each groups were presented as the *n* numbers. (B) RA-induced differentiation was performed by the regular method. Three days into the EB formation, 10 μM BrdU was added into the EB cultures for 12 h. BrdU incorporation was analyzed by immunostaining using an anti-BrdU antibody and a FITC-conjugated secondary antibody. BrdU-labeled cells were examined by flow cytometry. (C) On day 3 of RA-induced differentiation, EBs cell extracts were prepared and the caspase activities were determined by an in vitro caspase assay.

in the well-differentiated cells. Therefore, we examined whether restoration of UTF1 in P19-C8 cells affected earlier neuronal differentiation. We found that expression of UTF1 in P19-C8 cells also decreased, although did not abolish, the expression of nestin (an earlier neuronal marker) in RA-induced differentiation. This result indicated that UTF1 expression is sufficient to block the differentiation of neuronal cells, but not glial cells, in P19-C8 cells.

3.4. UTF1 deficiency up-regulates cell proliferation in EBs

Because abnormal EB formation hampers neuron differentiation (Hamada-Kanazawa et al., 2004; Wang et al., 2006), we examined the size of the EBs during differentiation process to investigate whether UTF1 deficiency affected EB formation. We cultured parental P19, C8 and C11 cells by the hanging drop method. After 6 days of aggregation, all cells formed EBs with normal morphology. Nevertheless, the average size of EBs of parental P19 cells was significantly smaller than those of P19-C8 and P19-C11 cells (Fig. 4A). We obtained consistent results from P19 cells treated with a UTF1 siRNA. EB aggregates from the UTF1-knockdown cells were significantly larger than those of control cells (Fig. 4A).

To investigate whether the larger EB formation in P19-C8 and C11 was resulted from an increase in cell proliferation, we used BrdU uptake to measure DNA replication in EBs. As shown in Fig. 4B,

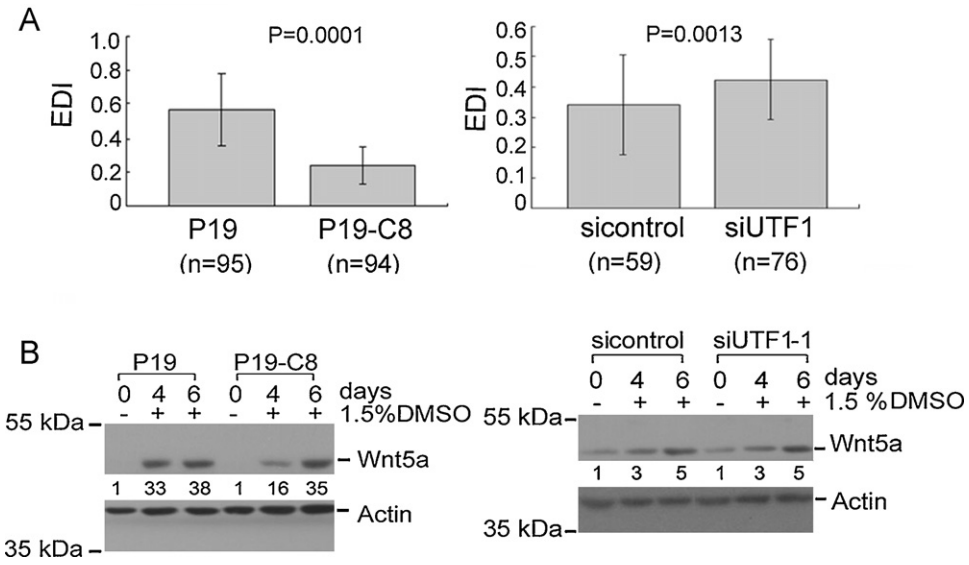

Fig. 5. UTF1 knockdown does not affect axial elongation in P19 EBs treated with DMSO. P19 cells (clones or siRNA-treated cells) were aggregated in hanging drops of DMEM medium containing 2% bovine serum and 1.5% DMSO. (A) The degree of elongated morphology in the cells aggregates in day 6 was presented by EDI as described in Section 2. The data were analyzed by unpaired, two-tailed Student's *t* test. The numbers of EBs analyzed in each groups were presented as the *n* numbers. (B) P19 cell lysates from the day 4 and day 6 aggregates were prepared and subjected to Western blotting analyses for Wnt5a and actin expression.

more cells are BrdU-positive in EBs formed by the UTF1-deficient clones than those of parental P19 cells. The increase of BrdU-positive cells was also found in EBs formed by P19 cells treated with siUTF1-1 (Fig. 4B). We also used a fluorogenic substrate, Ac-DEVD-AFC, to measure the caspase 3 activity in the EBs. No significant difference in the caspase activity was observed among P19-C8, P19-C11, and parental cells (Fig. 4C). Taken together, our data indicated that larger EB formation by UTF1-deficient P19 cells were resulted from an increase in cell proliferation rather than a decrease in apoptosis.

3.5. UTF1-deficient P19 clones were defective in mesoderm differentiation

Like mammalian embryos, P19 EBs show elongated morphology in long-term cultures. This axial elongation morphogenesis can be used as a criterion to investigate mesoderm differentiation (Marikawa et al., 2009). Accordingly, parental P19 and P19-C8 cells were subjected to hanging drop culture in the presence or absence of DMSO, a mesodermal differentiation agent. After 6 days, the cell aggregates showed elongated morphology if treated with DMSO and formed complete spheres in the absence of DMSO. An elongation distortion index (EDI) was used to quantitatively evaluate the degree of the axial elongation. Based on the EDI measurements, axial elongation in P19-C8 cells was significantly less than that in parental cells. However, knockdown of UTF1 by siRNA in P19 cells caused a minor increase of EDI (Fig. 5A). Because Wnt5a is essential for axial elongation of P19 cell EBs (Marikawa et al., 2009; Yamaguchi et al., 1999), we also examined the Wnt5a levels in EB aggregates. Compared to parental P19, the expression of Wnt5a was lower in EBs of P19-C8 cells on day 4 (Fig. 5B). The same experiment on P19-C11 cells showed similar results (Suppl. Fig. 5). However, suppression of UTF1 by siRNA alone did not affect the expression of Wnt5a (Fig. 5B). Therefore, our results indicated that UTF1-deficient P19 cells were impaired in the differentiation of axial mesoderm, but UTF1 deficiency alone may not be sufficient to cause this defect.

3.6. UTF1-deficient ES cells show impaired viability and an increase of neuro-ectoderm marker Gap43 upon differentiation

We found that transient transfection procedure hindered EB formation by ES cells. Therefore, we established permanent UTF1-deficient ES cells as described by van den Boom et al. (2007). ES cells proliferated poorly immediately followed the introduction of the shUTF1 vector, indicating that UTF1 was important for the viability of ES cells. The proliferation of ES-shUTF1 cells gradually recovered after weeks of culture (Fig. 6A). Expressing shUTF1 apparently decreased the level of UTF1, but not Oct4, in permanently transfected cells (Fig. 6B). We first examined the effect of shUTF1 on spontaneous ES cell differentiation. The expression of various lineage markers was examined by RT-PCR as previously described (van den Boom et al., 2007; Xu and Davis, 2010). We found that ES-shUTF1 cells have higher basal expression of endoderm markers (Sox17 and GATA4), but their induction after differentiation were not higher than those in control cells. The expression levels of the mesoderm marker Barchyury upon EB formation were comparable between ES-pSuper and ES-shUTF1 cells (Suppl. Fig. 6A). ES-shUTF1 cells showed an increase of the neuro-ectoderm marker Gap43 upon differentiation in comparison to that of ES-pSuper cells (Suppl. Fig. 6A and B). Additionally, under the RA-induced differentiation condition we found a similar increase of Gap43 in UTF1-deficient ES cells (Fig. 6C). These results suggested that UTF1 deficiency in ES cells also directed cells into the neuronal lineage.

3.7. UTF1-deficient P19 clones undergo neuronal differentiation without forming EBs

Forming EB is an essential step in directed or spontaneous differentiation of ES and EC cells. In light of the enhanced neuronal differentiation of P19 cells lacking UTF1, we tested whether UTF1-deficient P19 clones could undergo neuronal differentiation in the absence forming EBs. Seeding at a lower density (5×10^4/60-mm dish) in an adherent culture containing RA (5 μM), we found that the UTF1-deficient P19-C8 and C11 cells, but not parental P19 cells, underwent neuronal differentiation in 7 days. The neuronal

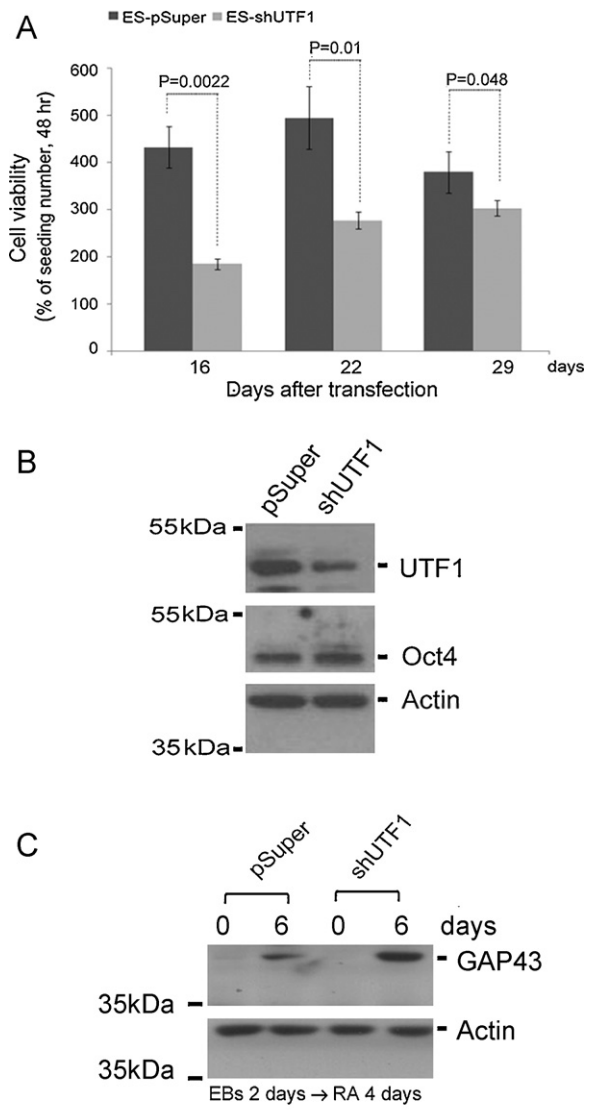

Fig. 6. UTF1-deficient ES cells show impaired viability and an increase of neuro-ectoderm marker Gap43 upon differentiation. ES-D3 cells were transfected with the pSuper vector or pSuper-shUTF1 for 48 h. Then the cells were subjected to puromycin (1 μg/ml) selection for permanently shUTF1-transfected cells. (A) On the indicated day post transfection, ES-pSuper and ES-shUTF1 cells were seeded at 1×10^5 cells/well, and the cell growth was monitored at the 48 h time point. The data presented are the means and standard deviations of representative triplicate experiments. The data were analyzed by unpaired, two-tailed Student's *t* test. (B) ES-pSuper and ES-shUTF1 cells over one-month selection were examined for their expression levels of UTF1, Oct4, and actin. (C) ES-pSuper and ES-shUTF1 cells over one-month selection were subjected to RA-induced differentiation, the expression levels and Gap43 and actin were examined by Western blot analyses.

differentiation could be detected morphologically or by Western blot analyses of βIII tubulin expression (Fig. 7A). The neuronal differentiation of P19-C8 and C11 cells were even more prominent when a defined N2/B27 medium was used in the culture system (Fig. 7B and C). Parental P19 cells showed no apparent neuronal differentiation in these culture conditions. The results indicated that, unlike parental P19, EB formation was no longer a required step for the neuronal differentiation of these UTF1-deficient cells.

4. Discussion

Suppression of UTF1 impairs ES cell proliferation while overexpression of UTF1 enhances it (Nishimoto et al., 2005; van den Boom et al., 2007). Moreover, introduction of UTF1, along with Oct4, Sox2, and Klf4, in human fibroblasts improves the efficiency of the generation of induced pluripotent stem cells (Zhao et al., 2008). These studies clearly show the significance of UTF1 in maintaining the pluripotency of ES cells. But how UTF1 regulates differentiation and whether it controls the development of all three embryonic germ layer lineages are still unclear.

In this study, we isolated UTF1-deficient cell clones P19-C8 and C11 from parental P19 cells. These clones showed lower expression levels of UTF1 but retained the expression of pluripotent markers Oct4 and Lin28. These UTF1-deficient P19 cells showed no defect in cell proliferation in adherent cultures. More interestingly, these UTF1-deficient P19 cells showed enhanced RA-induced neuronal differentiation in comparison to parental P19 cells. Suppression of UTF1 expression by specific siRNAs in P19 cells reproduced the same phenotype. Moreover, reconstitution of UTF1 expression in P19-C8 cells suppressed RA-induced neuronal differentiation. Our collected data implicated that the presence of UTF1 prevented neuronal differentiation of P19 cells. We found that the most prominent effect of UTF1 deficiency in ES cells was the impairment of cell proliferation. The cell proliferation ability was gradually recovered after weeks of cultures, indicating that ES cells might have rewired the regulatory mechanisms and adapted to the new condition. Under RA-directed or spontaneous differentiation conditions, we also observed a better expression of neuro-ectoderm marker Gap43 in ES-shUTF1 cells than that in control ES cells. However, whether this was a secondary effect resulted from the loss of viability remained to be determined. Different from the observations in ES cells, we found no apparent defects in cell proliferation of UTF1-deficient P19 cells maintained in adherent cultures. Consistent with our results, suppression of UTF1 expression in clonal P19-CL6 cells also does not suppress cell proliferation (van den Boom et al., 2007). We suspected that, different from ES cells, P19 EC cells might have overcome the dependence on UTF1 for their proliferation and survival.

We have also examined the effect of UTF1 in the regulation of mesoderm differentiation. In comparison to the parental P19 cells, UTF1-deficient P19 cells showed a decrease in mesodermal differentiation (axial elongation and Wnt5 expression); however, suppression of UTF1 alone was not sufficient to cause the same defects in P19 cells. These data indicated that deficiency in UTF1 alone might not be the reason for the defects in mesodermal differentiation. Our result is different from the report showing that suppression of UTF1 expression in P19 clone CL6 delays DMSO-induced mesodermal differentiation (van den Boom et al., 2007). This discrepancy could be resulted from the fact that P19-CL6 cell line, distinct from parental P19 cells, is isolated based on its tendency to differentiate into cardiac myocytes. Another possible explanation is that long-term deficiency of UTF1, such as that in P19-C8 and C11 cells, causes the defects in axial mesoderm differentiation through indirect mechanisms. In contrast, transient loss of UTF1, insufficient to cause the necessary secondary effects in short times, cannot affect the mesoderm differentiation of P19 EC cells.

It was bewildering to find that lacking UTF1 promoted the neuronal differentiation of P19 cells. The expression of UTF1 was greatly decreased in the first two days during RA-induced differentiation and the terminal differentiation of neurons (such as βIII tubulin expression and neurite extension) only occurred much later (4–6 days in the differentiation scheme). We have tried to elucidate the mechanism by which UTF1 deficiency promoted neuron differentiation. After examining the EB formation in a better-defined system (started with equal number of cells with hanging drop cultures), we found that UTF1-deficient cells (both natural clones and siUTF1-treated cells) formed larger EBs than did control P19 cells during RA-induced differentiation. In addition, BrdU uptake experiments confirmed that the UTF1-deficient cells and

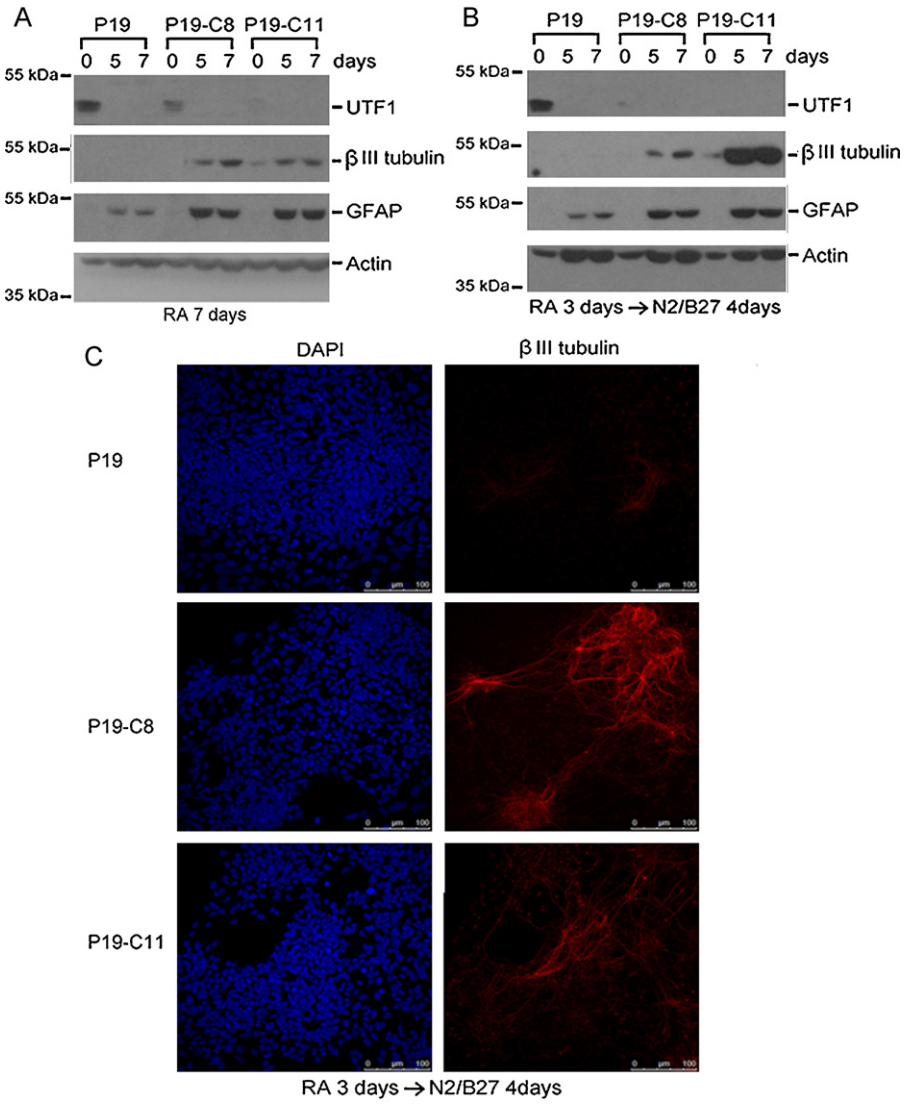

Fig. 7. UTF1-deficient P19 clones show obvious neuron differentiation in the absence of EB formation. (A and B) P19 cells (5×10^4/35-mm well) were treated with 5 μM of RA for 7 days (panel A) or treated with RA for 3 days and then were maintained in N2/B27 medium for another 4 days (panel B). The cells were collected at indicated time points and examined by Western blotting using UTF1, βIII tubulin, GFAP, and actin antibodies. (C) Neuronal differentiation of cells cultured in N2/B27 medium was examined by immunofluorescence staining using an anti-βIII tubulin antibody. Cell nuclei were stained by DAPI. The scale bars indicate the length of 100 μm.

UTF1 knockdown cells showed increased DNA replication in EB aggregates. Although treating cells with RA causes cell apoptosis (Okazawa et al., 1996; Fujita et al., 1999; Lee et al., 2006), we found that P19 parental cells and UTF1-deficient clones had comparable levels of caspase activity in their EBs. These data indicated that increased sizes of EBs in the UTF1-deficient cells were caused by an increase in cell proliferation rather than a decrease in apoptosis. Thus, we propose that loss of UTF1 predispose P19 cells in a state that allows better proliferation in EBs. Interestingly, a recent study shows that neuronal spheres formed by Sox1-deficient neuronal stem cells also have an increase in cell proliferation. The neuronal spheres also show better neuronal differentiation after plating. Sox1 regulates neuronal differentiation through suppressing the expression of Prox1, a homeodomain-containing regulator for neurogenesis (Elkouris et al., 2011). The possible relationship among UTF1, Sox1, and Prox1 deserves further examined.

Other than the enhanced cell proliferation in EBs, we found that UTF1-deficient P19 clones could differentiate into neuronal cells in the absence of EB formation. Aggregation of EC or ES cells is an essential step for spontaneous or agent-directed differentiation into various cell lineages. It has been suggested that aggregation and EB formation provide a cue(s) that may mimic the differentiation signal(s) occur in intact embryos (Doetschman et al., 1985; Dushnik-Levinson and Benvenisty, 1995; Itskovitz-Eldor et al., 2000). Our result implicated that deficiency in UTF1 might have removed a hurdle(s) for neuronal differentiation and bypassed the need for EB formation. Because of their readiness in RA-induced neuronal differentiation in vitro, these UTF1-deficient P19 clones may serve as additional cell models for researches of neuronal differentiation and development.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2011.11.008.

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