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Synergistic action of Wnt and LIF in maintaining pluripotency of mouse ES cells

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Abstract

Leukaemia inhibitory factor (LIF) was the first soluble factor identified as having potential to maintain the pluripotency of mouse embryonic stem (ES) cells. Recently, a second factor, Wnt, with similar activity was found. However, the relationship between these completely different signals mediating the overlapping functions is still unclear. Here, we report that the conditioned medium of L cells expressing Wnt3a maintains ES cells in the undifferentiated state in feeder-free culture, followed by expression of stem cell markers and their ability to generate germline chimaeras. However, although the activity of this conditioned medium is dependent on Wnt3a, recombinant Wnt3a protein cannot maintain ES cells in the undifferentiated state. As supplementation with Wnt3a to the sub-threshold level of LIF alone was not sufficient to maintain ES self-renewal, the results of maintenance of the undifferentiated state indicated the synergistic action of Wnt and LIF. Induction of constitutively activated β-catenin alone is unable to maintain ES self-renewal but shows a synergistic effect with LIF. These observations indicate that the Wnt signal mediated by the canonical pathway is not sufficient but enhances the effect of LIF to maintain self-renewal of mouse ES cells.

Keywords: Embryonic stem cell; Self-renewal; Wnt; LIF; β-Catenin; BIO

Mouse ES (mES) cells can be propagated in medium containing foetal calf serum (FCS) and the cytokine, leukaemia inhibitory factor (LIF), without the support of feeder cells [1,2]. The effect of LIF is mediated through a cell-surface complex composed of LIFRβ and gp130. Upon ligand binding, gp130 activates Janus-associated tyrosine kinases (JAK) and their downstream component, signal transducer and activator of transcription (STAT)3. Activation of STAT3 is necessary and sufficient for suppression of differentiation of mES cells [3,4]. However, LIF alone is not sufficient for clonal expansion of feeder-free mES cells in the absence of serum [5] and is unable to support self-renewal of human or monkey cells without feeder layers even in the presence of serum [6,7]. These findings suggest that unidentified growth factors provided by feeder cells and serum contribute to the maintenance of the self-renewal capacity of ES cells.

Wingless/Wnts are developmentally regulated secretory proteins that control cell differentiation, movement, and proliferation. Large-scale gene expression profiling of mES cells revealed that components of several signal transduction pathways are transcriptionally enriched in the undifferentiated state, and the main components of the canonical Wnt pathway are detected in ES cells in the undifferentiated state [8]. Sato et al. [9] reported that the GSK-3 inhibitor, 6-bromoindirubin-3′-oxime (BIO), maintains the pluripotency of mES and human ES (hES) cells. However, another group reported that addition of Wnt3a stimulated not only hES cell proliferation but also

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differentiation [10]. These discrepancies suggest that the canonical Wnt signal activation is not absolutely sufficient for maintenance of the pluripotency of ES cells, but that its activity is context-dependent.

In this study, we investigated the effects of the Wnt signal on mES cell self-renewal using a stem cell selection system based on Oct3/4 expression and feeder-free culture conditions [11], which allowed us to determine the direct effect of Wnt on undifferentiated ES cells.

Materials and methods

ES cell culture. ES cells were maintained on feeder-free gelatine-coated plates in FCS-containing medium supplemented with LIF: Glasgow minimal essential medium (GMEM; Sigma–Aldrich) supplemented with 10% FCS (selected batches, Sigma–Aldrich), 100 μM 2-mercaptoethanol (Nacalai Tesque), 1× non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 1000 U/ml (U) LIF (ESGRO; Invitrogen). EB3 and OLG2-3 ES cells were generated by introduction of Oct3/4 knockout vector carrying IRESBSDpA and EGFP IRES pacpA into E14TG2a ES cells by homologous recombination [11,12], and cultured in the presence of 5 μg/ml blasticidin S (Kaken Pharmaceutical) and 1.5 μg/ml puromycin (Sigma–Aldrich) for stem cell selection. For in vitro experiments, EB3, OLG2-3 or D3 ES cells were seeded onto gelatine-coated 12- or 6-well plates at a density of 3×10^2 or 1×10^3 cells/well, and cultured for 5–6 days in conditioned or non-conditioned medium, in the presence of LIF or recombinant Wnt3a (R&D Systems) or recombinant frizzled8-Fc (Fz8-Fc; R&D Systems) or anti-LIF antibody (R&D Systems), which were added to fresh medium three days later, or the GSK-3 inhibitor, 6-bromoindirubin-3′-oxime (BIO; Calbiochem). Alkaline phosphatase staining was carried out using BCIP/NBT solution (Sigma-Aldrich).

Conditioned media. L cells (1×10^6 cells) expressing mouse Wnt3a or vector alone (ATCC) were plated onto dishes 9 cm in diameter [13]. Three days later, cells were washed three times with phosphate-buffered saline (PBS) and transferred to fresh FCS-containing medium. The supernatants were collected after 3 days and used at a threefold dilution for ES cell culture. These conditioned media were designated L-Wnt3a CM and L-CM, respectively. To collect the supernatants of L cells stimulated with Wnt-3a, L cells (1×10^6 cells) were expanded in L-Wnt3a CM for 3 days, washed three times with PBS, and transferred to fresh FCS-containing medium. The supernatants were collected after 3 days and designated L(w3a) CM, and used at a threefold dilution for ES cell culture.

Generation of EBRTcPβ-cateninwt and EBRTcPβ-cateninΔGSK ES cells. β-Catenin and β-cateninΔ*GSK* cDNA were introduced into the *Xho*I and *Not*I sites of the exchange vector pPthC to obtain pPthC-β-cateninwt and pPthC-β-cateninΔ*GSK*, respectively [14,15]. EBRTcH3 ES cells were seeded onto gelatine-coated 6-well plates at a density of 1×10^5 cells/well. The next day, 0.5 μg pPthC-β-cateninwt or pPthC-β-cateninΔ*GSK* and 0.5 μg pCAGGS-Cre were co-transfected using Lipofectamine 2000 (Invitrogen). The transfected cells were re-plated onto dishes 9 cm in diameter in medium containing 1 μg Tc (Sigma–Aldrich) and transferred to medium FCS-containing 1 μg Tc and 1.5 μg/ml of puromycin. The selected recombinant cells were maintained in FCS-containing medium containing 1 μg Tc and 1.5 μg/ml of puromycin. For in vitro experiments, EBRTcPβ-cateninwt or EBRTcPβ-cateninΔGSK ES cells were seeded onto gelatine-coated 6-well plates at a density of 2×10^3 cells/well, and cultured for 5 days in FCS-containing medium, in the presence or absence of Tc or LIF, which were added to fresh medium three days later. Alkaline phosphatase staining was carried out using BCIP/NBT solution (Sigma–Aldrich).

Luciferase reporter assay. OLG2-3, EBRTcPβcateninwt, and EBRTcPβcateninΔGSK ES cells were seeded onto gelatine-coated 24-well plates at a density of 5×10^4 cells/well. The following day, cells were co-transfected with 0.5 μg of TOP-Flash or FOP-Flash reporter (Upstate) or 2 μg of 4 × APRE-luc and 0.005 μg or 0.02 μg pRL-CMV (Promega) using Lipofectamine 2000 [16,17]. Luciferase activities were measured by dual luciferase assay 24 h after transfection (Promega). Luciferase activities were normalized to those of co-transfected pRL-RL.

RNA isolation, Northern blotting, and reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNAs were prepared with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Oligo(dT)-primed cDNAs were prepared from 1 μg of total RNA using ReverTra Ace (Toyobo) and aliquots of 1/20th of the cDNA products were used for each PCR amplification. The gene-specific primers were as follows: sense primer for *H19* CAAGGTGAAGCTGAAAGAACA GATGG, antisense primer for *H19* TCCAAACCAGTGCAATCGACTT AG, sense primer for *tPA* GCCCTCTGGTGTGCATGATCAAT and antisense primer for *tPA* TTCCAAAGCCAGACCTTCATCCTT. These corresponded to the Accession Nos: J03250 (*tPA*) and X58196 (*H*19). PCR products were separated by electrophoresis on 1.2% agarose gels and visualized with ethidium bromide. For Northern blotting analysis, aliquots of 4 μg of total RNA were separated on denaturing agarose gels and then blotted onto Hybond-N membranes (Amersham Biosciences). Analyses were performed with GeneImage (Amersham Biosciences) according to the manufacturer's instructions.

Generation of chimaeric mice. EB3 cells were propagated at clonal density and maintained for more than 2 months in L-Wnt3a CM. Microinjection of ES cells into C57BL/6J blastocysts was performed according to standard procedures [18].

Western blotting analysis. For analysis of STAT3 phosphorylation, aliquots of 1×10^7 OLG2-3 ES cells were seeded onto gelatine-coated dishes 9 cm in diameter and cultured in FCS-containing medium without LIF. The next day, cells were treated with LIF or Wnt3a in FCS-containing medium for 2 h. Cells were then washed twice with ice-cold PBS and scraped off in 100 μl of ice-cold lysis buffer (50 mM Tri–HCl, pH 7.4, 1% NP-40, 0.025% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, and 1 mM NaF) containing 1% proteinase inhibitor cocktail (Sigma). Aliquots of 30 μg of total protein from each sample were separated by electrophoresis on 10% SDS–polyacrylamide gels and electroblotted onto PDF membranes (Immobilon; Nihon Millipore). After treatment in blocking buffer (10 mM Tris–HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, and 3% skimmed milk), the membranes were probed sequentially with anti-STAT3 (Cell Signaling Technology) or anti-phospho-STAT3 (705Tyr) (Cell Signaling Technology) and then with horseradish peroxidase-coupled anti-rabbit IgG (Jackson Immuno Research), developed using ECL reagents (Amersham Biosciences).

Results and Discussion

To determine whether activation of the Wnt signal is absolutely sufficient to maintain pluripotency of mES cells, we first examined the effects of conditioned medium from mouse fibroblast L cells expressing Wnt3a (L-Wnt3a CM) as a source of Wnt activity on mES self-renewal using a feeder-free culture system [11,13]. To determine the actual effect of Wnt3a in the conditioned medium on mES cells, we used two different negative controls: conditioned medium from wild-type L cells (L CM) and conditioned medium from these cells stimulated with L-Wnt3a CM (L(w3a) CM). The latter allowed us to distinguish the direct effect of Wnt3a on mES cells from its indirect effect via stimulation of L cells to produce factors that affect mES self-renewal. Monitoring of Wnt activity on mES cells by luciferase assay using TOP-Flash (TOP) and FOP-Flash (FOP) reporters revealed that only L-Wnt3a CM contained significant levels of Wnt activity sufficient to stimulate the canonical Wnt signal mediating β-catenin/T-cell-specific factor (TCF) transcriptional activity in mES cells

(Fig. 1A) [16]. In contrast, the same reporter assay revealed no detectable activation of the canonical Wnt signal in mES cells maintained by LIF, indicating that LIF does not have any cross-reactivity either directly or indirectly via activation of the autocrine loop of the Wnt signal. Functional assays of these conditioned media indicated that only L-Wnt3a CM showed the ability to maintain EB3 or OLG2-3 feeder-free ES cells in the undifferentiated

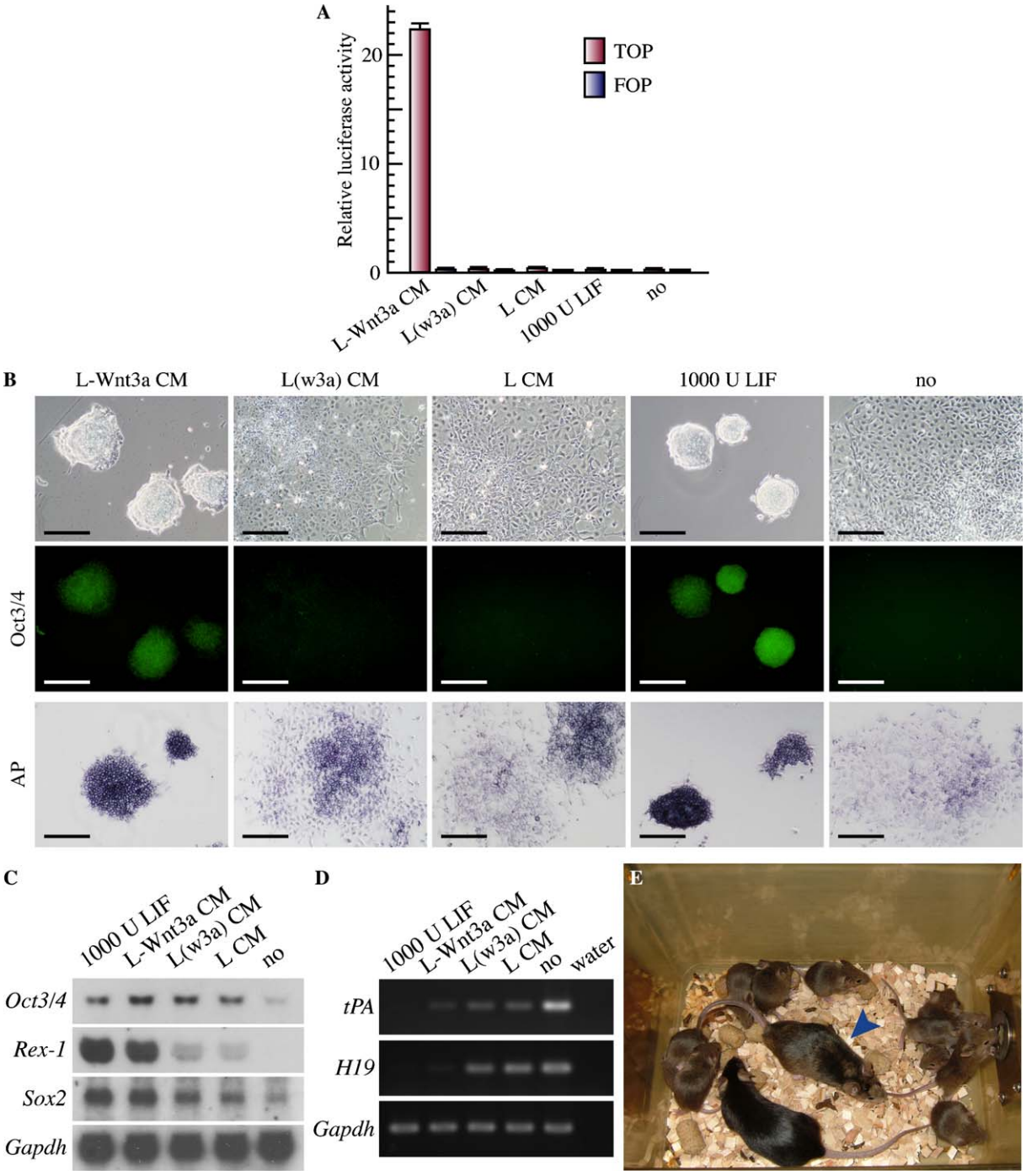

Fig. 1. L-Wnt3a CM maintains the pluripotency of mES cells. (A) Luciferase assay for TOP/FOP reporters in OLG 2-3 ES cells. Each bar represents the mean ± SEM ($n = 3$). (B) Colony morphologies of OLG2-3 and EB3 ES cells. OLG2-3 (upper and middle) or EB3 (lower) cells were cultured in L-Wnt3a CM, L(w3a)CM, L CM, or FCS-containing medium supplemented with or without LIF for 5 days. The middle panel shows expression of Oct3/4-EGFP and the lower panel shows AP-staining as markers of the undifferentiated state. Scale bars, 200 μm. (C) Expression of stem cell marker genes *Oct3/4*, *Sox2*, and *Rex1* in OLG2-3 ES cells cultured in L-Wnt3a CM, L(w3a)CM, L CM or FCS-containing medium supplemented with or without LIF for 5 days examined by Northern blotting analysis. *Gapdh* was used as a loading control. (D) Expression of differentiated cell marker genes in OLG2-3 ES cells analysed by RT-PCR. *Gapdh* was used as loading control. (E) Germline-competent male chimaeric mice (arrowhead) derived from EB3 cells cultured with L-Wnt3a CM for at least 2 months, and mated with female C57BL/6J mice, resulting in Agouti pups.

state, as determined by the compact colony morphology as well as the expression of Oct3/4 or alkaline phosphatase (AP) activity, comparable to that of medium containing 1000 U/ml (U) of recombinant LIF (Fig. 1B). Another cell line, D3 ES cells, can also be maintained in L-Wnt3a CM (Fig. 2D). Maintenance of the undifferentiated state and prevention of differentiation were confirmed by the expression of marker genes, and stem cell markers, such as *Oct3/4*, *Rex1*, and *Sox2*, were maintained (Fig. 1C), while differentiation markers, such as *tPA* and *H19*, were suppressed in ES cells maintained in L-Wnt3a CM (Fig. 1D) [11,19,20]. Finally, the appropriate maintenance of pluripotency in L-Wnt3a CM was confirmed by generation of germline-competent chimaeric mice by injection of EB3 ES cells maintained in L-Wnt3a CM for over 2 months (Fig. 1E). These results indicated that L-Wnt3a CM is absolutely sufficient to maintain pluripotency of mES cells and that Wnt3a in L-Wnt3a CM contributes directly to this effect.

Next, we examined the effect of Wnt3a on mES cells as distinct from that of L-Wnt3a CM. First, we evaluated the contribution of Wnt3a to the effect of L-Wnt3a CM by withdrawal of its activity using soluble frizzled receptor, Fz8-Fc, a specific Wnt antagonist. Although the almost complete blockage of Wnt3a activity by Fz8-Fc was monitored by TOP/FOP reporter assay (Fig. 2A), we found that the effect of Fz8-Fc on ES self-renewal maintained by L-Wnt3a CM was weaker than that of complete replacement of L-Wnt3a CM with L CM (Figs. 1B and 2B), suggesting that not only Wnt3a but also another factor(s) contributes to the ability of L-Wnt3a CM to maintain ES self-renewal. As LIF is the most potent factor to maintain pluripotency of mES cells, we tested the activity of LIF in Wnt3a CM by LIF-dependent reporter assay. Experiments using a STAT3-responsive reporter 4×acute phase response element (APRE)-luc indicated that Wnt3a CM contains LIF activity comparable to that of 10 U of recombinant LIF (Fig. 3C) [17]. The concentration of LIF in L-Wnt3a CM was estimated as 60 pg/ml (about 6 U) by enzyme immunoassay, whereas that in L CM was 20 pg/ml (about 2 U). Neutralization of LIF activity in L-Wnt3a CM by addition of anti-LIF antibody resulted in its reduced ability to maintain self-renewal (Fig. 3D). These observations suggested that L-Wnt3a CM may maintain the pluripotency of mES cells via the synergistic action of Wnt3a and LIF.

The synergistic action of Wnt3a and LIF on mES self-renewal was evaluated by addition of each recombinant protein to serum-containing medium. Activation of STAT3 by phosphorylation on 705Tyr was reported to be necessary and sufficient to maintain pluripotency of mES cells [3]. Addition of recombinant Wnt3a resulted in activation of the canonical Wnt signal monitored by TOP/FOP reporters comparable to that by L-Wnt3a CM (Fig. 3A), but had no effect on STAT3 phosphorylation (Fig. 3B), whereas recombinant LIF stimulated STAT3 phosphorylation in a dose-dependent manner without activation of the canonical Wnt signal (Figs. 3A and B). These observations indicated that there is no cross-effect between these two ligands. Various concentrations of LIF were examined

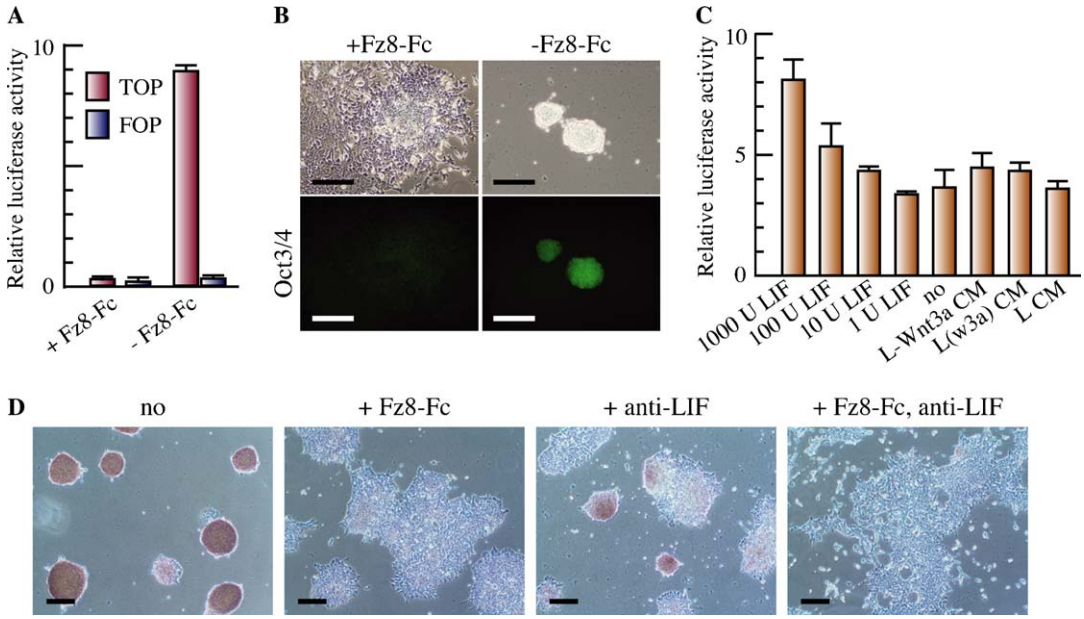

Fig. 2. Synergistic effect between Wnt and LIF signal in L-Wnt3a CM. (A) Luciferase assay for TOP/FOP reporters in OLG 2-3 ES cells. Each bar represents the mean ± SEM ($n = 3$). (B) Effects of antagonists against Wnt (1 μg/ml mouse Fz8-Fc) on self-renewal of OLG2-3 ES cells cultured in L-Wnt3a CM. ES cells were cultured in L-Wnt3a CM supplemented with (left) or without Fz8-Fc (right) for 5 days. The lower panel shows expression of Oct3/4-EGFP. Scale bars, 200 μm. (C) Luciferase assay for 4 × APRE-luc in OLG2-3 ES cells. (D) Effects of antagonists against Wnt (1 μg/ml mouse Fz8-Fc) and/or LIF (1 μg/ml anti-LIF antibody) on self-renewal of D3 ES cells cultured in L-Wnt3a CM. D3 ES cells were cultured in L-Wnt3a CM supplemented with Fz8-Fc and/or anti-LIF antibody or with no additional factors for 6 days. AP staining shows undifferentiated D3 ES cells.

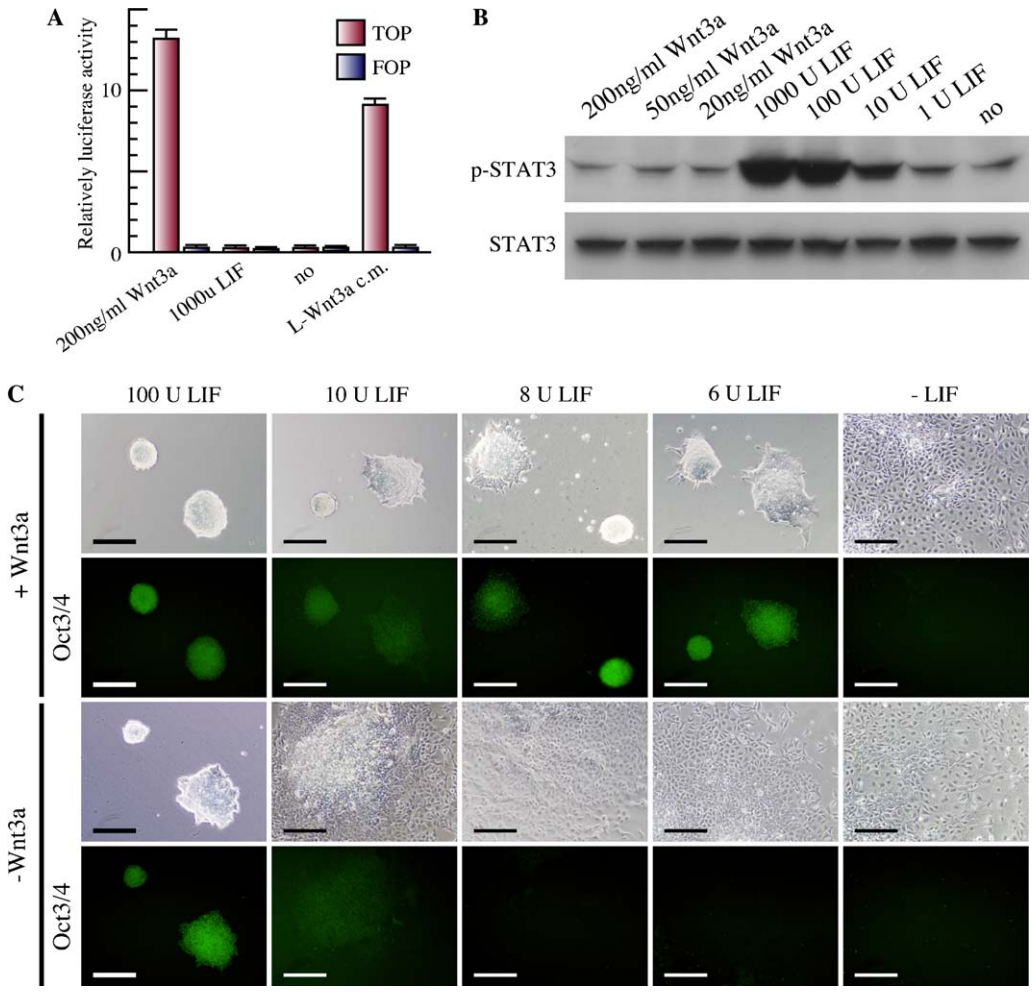

Fig. 3. Synergistic effect between Wnt3a protein and LIF on mES cell self-renewal. (A) Luciferase assay for TOP/FOP reporters in OLG2-3 ES cells. Each bar represents the mean ± SEM ($n = 3$). (B) Effects of Wnt3a protein on STAT3 phosphorylation in OLG2-3 ES cells. EB5 cells were treated with Wnt or LIF at the indicated concentration for 2 h and subjected to Western blotting analysis using anti-phospho-STAT3 (705Tyr) antibody (upper panel) and anti-STAT3 antibody (lower panel). (C) Colony morphologies of OLG2-3 ES cells. OLG2-3 cells were cultured in FCS-containing medium supplemented with or without 200 ng/ml Wnt3a at various concentrations of LIF for 5 days. The second and fourth panels show expression of Oct3/4-EGFP. Scale bars, 200 μm.

for their abilities to maintain mES cells in the undifferentiated state. Our results indicated that 10 U was the minimal dose to give Oct3/4-positive stem cell colonies (Fig. 3C). In contrast, if recombinant Wnt3a was added simultaneously with LIF, 6 U of LIF was sufficient to support Oct3/4-positive colony formation (Fig. 3C), indicating that Wnt3a can reduce the requirement for LIF to maintain self-renewal. However, Oct3/4-positive colonies could not be generated in medium containing Wnt3a without LIF (Fig. 3C), indicating that Wnt3a alone is not sufficient to maintain ES self-renewal but that it acts synergistically with LIF.

These observations raise the question of how the synergistic action of Wnt3a and LIF is mediated. To assess the contribution of the canonical Wnt signal to this phenomenon, we examined the effect of direct activation of this signal by accumulation of β-catenin in ES cells. For this purpose, we introduced tetracycline-regulatable transgenes for expression of either wild-type (wt) or the constitutively active form of β-catenin, which carries four point mutations (S33A, S37A, T41A, and S45A) at the GSK-binding site to prevent the interaction [14,15]. The effect of β-catenin ΔGSK overexpression on the canonical Wnt signal was confirmed by TOP/FOP reporters, which was comparable to the effect mediated by L-Wnt3a CM (Fig. 4A). The effects of overexpression of β-catenin wt and β-catenin ΔGSK to support self-renewal, and we detected their synergistic activities with sub-threshold levels of LIF to maintain the cells in the undifferentiated state (Fig. 4B). Moreover, as shown in the case of recombinant Wnt3a, direct activation of the canonical Wnt signal alone was not sufficient to maintain ES self-renewal. These results suggested that the canonical Wnt signal acts synergistically with the LIF signal.

Sato et al. [9] reported that the GSK3β-specific inhibitor, BIO, can replace the activity of L-Wnt3a to maintain ES self-renewal. Therefore, we examined its effect using our feeder-free ES cell culture system as described for L-Wnt3aCM and recombinant Wnt3a. Interestingly, addition of BIO to

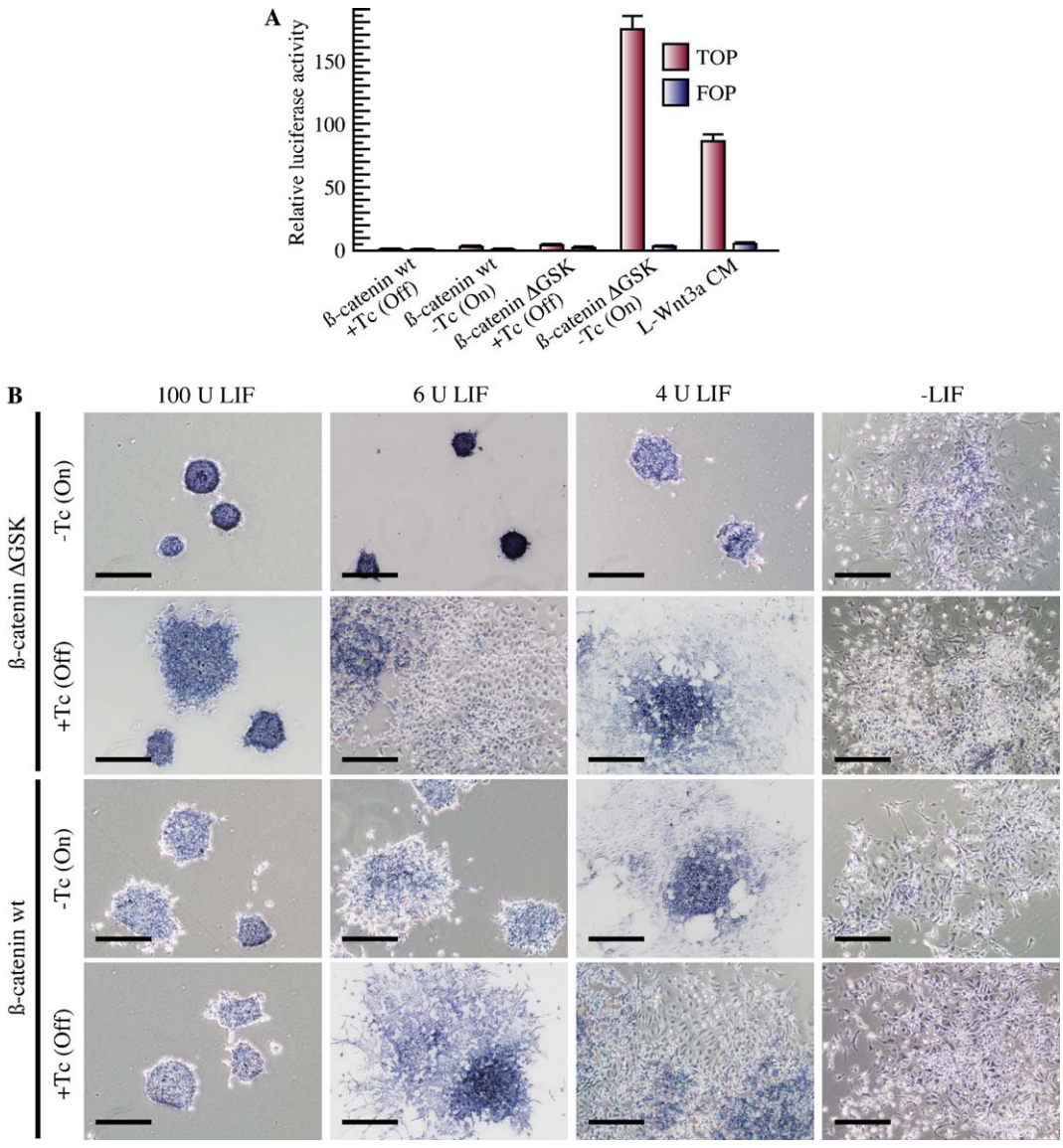

Fig. 4. Synergistic effect between constitutively activated β-catenin induction and LIF on mES cell self-renewal. (A) Luciferase assay for TOP/FOP reporters in EBRTcPβ-catenin wt and EBRTcPβ-cateninΔGSK ES cells. Each bar represents the mean ± SEM ($n = 3$). (B) AP-stained colony morphologies of EBRTcPβ-catenin wt (upper panel) and EBRTcPβ-cateninΔGSK (lower panel) ES cells. Both ES cells were cultured in FCS-containing medium supplemented with or without Tc at various concentrations of LIF for 5 days. Withdrawal of Tc (top and third panels) induced expression of β-catenin wt and β-catenin ΔGSK. Scale bars, 200 μm.

FCS-containing medium resulted in not only strong activation of the canonical Wnt signal as determined using TOP/FOP reporters (Fig. 5A), but also generation of Oct3/4-positive colonies in the absence of LIF, although the colony size was smaller than that supported by LIF for the same period (Fig. 5C). The effect of BIO on the LIF signal was estimated using APRE-luc, and the results indicated that BIO activated its expression to the same level as that induced by 100 U LIF (Fig. 5B). Although the molecular mechanism of this effect remains unclear, maintenance of ES self-renewal by BIO may be due to its combinatorial effect in activating both the canonical Wnt signal and the LIF signal simultaneously, as in the case of L-Wnt3a CM and the combination of recombinant Wnt3a and LIF.

The results of the present study indicated that addition of recombinant Wnt3a protein and induction of constitutively activated β-catenin maintain the pluripotency of mES cells by cooperation with sub-threshold levels of LIF, but that they were not sufficient to maintain self-renewal of mES cells in the absence of LIF. Our findings in mES cells were consistent with those in mouse haematopoietic stem (HS) cells, for which Wnt3a by cooperation with Steel locus factor (SLF) has been shown to support stem cell self-renewal [21]. These findings indicate that the function of the Wnt signal is limited to a supportive effect for the maintenance of the pluripotency of mES cells.

We found that BIO maintained the undifferentiated state of a subset of mES cells and induced differentiation

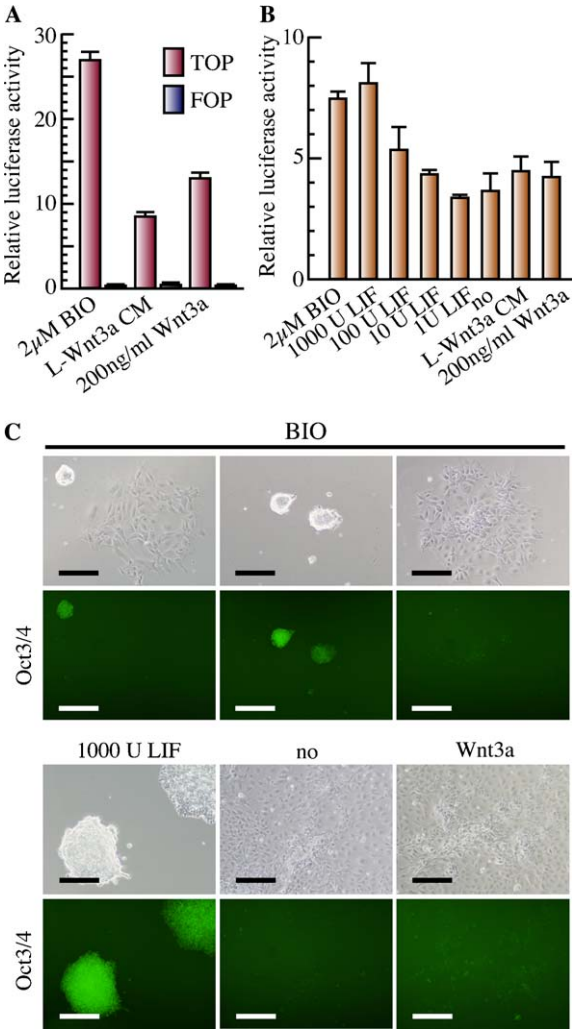

Fig. 5. Synergistic effect between Wnt and LIF signal by BIO. (A) Luciferase assay for TOP/FOP reporters in OLG 2-3 ES cells. (B) Luciferase assay for 4 × APRE reporters in OLG 2-3 ES cells. Each bar represents the mean ± SEM ($n = 3$). (C) Effects of BIO on OLG2-3 ES cell self-renewal. ES cells were cultured in FCS-containing medium supplemented with 2 μM BIO, 1000 U LIF, 200 ng/ml Wnt3a or no factor for 6 days. The lower panel shows expression of Oct3/4-EGFP. Scale bars, 200 μm.

of the remaining cells with activation of STAT3-specific reporter. The variation of responsiveness of mES cells to BIO may reflect the heterogeneity of Oct3/4-positive mES cells in which different subpopulations show various capabilities of signal integration [22]. Our results suggest that BIO may have combinatorial effects on mES cells, activating both the canonical Wnt signal and the LIF signal simultaneously to maintain the cells in the undifferentiated state, as in the case of the combination of recombinant Wnt3a and LIF. We could not evaluate the mechanism by which BIO activates STAT3 transcriptional activity as there is no evidence of a connection between GSK3 and the LIF-Jak-STAT3 signal cascade.

It was reported recently that recombinant Wnt3a alone is not sufficient to maintain human ES cells in the undifferentiated state, although L-Wnt3a CM can do so [9,10]. These findings were similar to our observations in mES cells, indicating that factor(s) in L-Wnt3a CM act synergistically with Wnt. However, it was also reported that activation of STAT3 is neither sufficient nor necessary to maintain self-renewal of hES cells. Therefore, the LIF-STAT3 signal should not be a partner of Wnt for its synergistic action. We are currently attempting to identify the unknown partner(s) of Wnt for human ES cells, which may be activated by BIO, as it is sufficient to maintain the undifferentiated state in a limited period.

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