厚生労働科学研究費補助金（免疫アレルギー疾患等予防・治療研究事業）
分担研究報告書

細胞レベルでの免疫学的研究

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研究要旨：
移植片に対する免疫寛容の機序を細胞レベルで解明することを目指して、本研究ではモデル実験系を確立して解析を進めている。実験系としては、タンパク抗原 OVA とスーパー抗原 TSST-1 と反応する TCR 遺伝子導入の T 細胞クローンマウス(TCR Tg)を用い、昨年度は TSST1 によりアナジー様 T 細胞を誘導した。本年度は、それらアナジー様 T 細胞が刺激を受けていない in tact の T 細胞による活性化を in vitro で抑制することが示唆された。これらの活性化抑制の機序を解明する系として、TCR Tg に GFP Tg を交配させた。得られた F1 マウスの T 細胞は、TCR Tg 単独の場合と同様に TSST-1 によりアナジー様 T サイボウが誘導された。これらマウスでは、アナジー様 T 細胞と初回活性化を受けた抗原特異的 T 細胞を識別しすることが可能となり、in vitro のみならず in vivo における活性化 T 細胞の動態を解析可能とする。

A．研究目的

免疫反応が抗原特異的に抑制される現象は免疫寛容として古くから知られているが、その機序は不明のままである。この点を解明することは、移植生着に有用な知見をもたらすと考え、本研究では免疫抑制を誘導できるできるだけ単純な実験系を確立する

B．研究方法

我々が樹立した卵白アルブミン（OVA）と反応する T 細胞受容体(TCR)遺伝子導入マウス（OVA-TCR Tg）はタンパク抗原 OVA の他にスーパー抗原 TSST-1 にも反応する。TSST-1 で再刺激を受けた T 細胞を、無刺激 T 細胞と OVA/TSST-1 の存在下で co-culture し、新しく刺激された T 細胞の活性化状態を、増殖性サイトカイン IL-2 と抑制サイトカイン①IL-10 産生能を ELISA および real time PCR で、②活性化マーカーの発現（Flow-cytometer）にて解析する。また、すでにアナジー状態となっている T 細胞と新たに刺激された T 細胞を識別するために、OVA-TCR Tg と C57BL6(B6)背景の(b-actin-GFP) Tg マウスを交配する。
倫理面への配慮に関しては、マウス細胞のみを用いたため必要としなかった。

C．研究結果

①無刺激の脾臓細胞をTSST-1刺激(in vivo系)により誘導したアナジー状態のT細胞とco-cultureすると、OVAに対してもTSST-1に対しても単独培養の場合よりIL-2産生能は低下していた。この傾向はOVA刺激の場合により強かった。この結果は、sAgにより誘導されたアナジーT細胞が同一TCRを介した新しいクローンの活性化を阻止しうることを示唆している。②新たなT細胞クローンの活性を抑制したのがアナジー状態となっているT細胞であるのか、またその機序を検討するために、(OVA-TCR x GFP)Tgマウスを作製した。OVA-TCR TgはBALB/c/B6背景で維持しているため、B6由来のGFP Tgとの一回の交配で２５％の使用可能なマウスを得、それらを実験用に可能な個体数を得るためにほぼ１０ヵ月を要した。それらマウスは、TSST-1によるin vivo刺激でアナジー様状態の細胞を誘導できることを確認した。これらマウスは、またin vivoにおけるアナジーT細胞の抑制機能を解析するため、現在繁殖を拡大している。

D．考察

上記の結果、in vitro の実験系では、TSST-1 によって誘導された T 細胞のアナジー状態の T 細胞は周辺の刺激を受けた in tact の T 細胞クローンの活性化を抑制することが示唆された。移植における寛容現象誘導に繋げるためには、上記アナジー様細胞の in vivo に置ける抑制機能の検証が必要である。そのために、本研究で使用している TCR Tg を GFP Tg と交配・繁殖させて、対移片反応性 T 細胞の活性化の動態を追跡できる準備が整った。本 F1 マウスはまたいわゆる Treg 細胞の分化を解析する上でも有用であると考え、来年度の計画に組み込む予定である。

E．結論

同一 TCR を介した２種類の異なる抗原刺激（通常タンパクおよびスーパー抗原）を使い分けることによって、後者ではアナジー様 T 細胞が誘導され、前者の活性化を抑制することを示唆する in vitro のデータを得た。アナジー様 T 細胞が活性

化 T 細胞を抑制する機序を in vitro と in vivo で解明するため、TCR Tg と GFP Tg を交配した F1 マウスを作製した。

F．健康危険情報
総括研究報告書参照

G．研究発表
1. 論文発表

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4) Th2 immune response player a critical role in the development of nickel-induced　llergic contanct dermatitis.　Niiyama S, Tamauchi H, Amoh Y, Terashima M, Matsumura Y, Kanoh M, Habu S, Komotori J, Katsuoka K. Int Arch Allergy Immunol, 153:303-314, 2010

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H．知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
　なし
2. 実用新案登録
　なし
3. その他
　なし

III. 研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表レイアウト

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Wada H, Kojo S, Kusama C, Okamoto N, Sato Y, Ishizuka B, **Seino K**.	Successful differentiation to T cells, but unsuccessful B-cell generation, from B-cell-derived induced pluripotent stem cells.	Int. Immunol.	23	65-74	2011
Abe N, Hozumi K, Hirano K, Yagita H, **Habu S**	Notch ligands transduce different magnitudes of signaling critical for determination of T-cell fate.	Eur J Immunol	40	2608-2617	2010
Wong WF, Nakazato M, Watanabe T, Kohu K, Ogata T, Yoshida N, Sotomaru Y, Ito M, Araki K, Telfer J, Fukumoto M, Suzuki D, Sato T, Hozumi K, **Habu S**, Satake M	Over-expression of Runx1 transcription factor impairs the development of thymocytes from the double-negative to double-positive stages.	Immunology	130	243-253	2010
Niiyama S, Tamauchi H, Amoh Y, Terashima M, Matsumura Y, Kanoh M, **Habu S**, Komotori J, Katsuoka K	Th2 immune response plays a critical role in the development of nickel-induced allergic contact dermatitis.	Int Arch Allergy Immunol	153	303-314	2010

IV. 研究成果の刊行物・別刷

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Successful differentiation to T cells, but unsuccessful B-cell generation, from B-cell-derived induced pluripotent stem cells

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Abstract

Forced expression of certain transcription factors in somatic cells results in generation of induced pluripotent stem (iPS) cells, which differentiate into various cell types. We investigated T-cell and B-cell lineage differentiation from iPS cells *in vitro*. To evaluate the impact of iPS cell source, murine splenic B-cell-derived iPS (B-iPS) cells were generated after retroviral transduction of four transcription factors (Oct4, Sox2, Klf4 and c-Myc). B-iPS cells were identical to embryonic stem (ES) cells and mouse embryonic fibroblast (MEF)-derived iPS cells in morphology, ES cell marker expression as well as teratoma and chimera mouse formation. Both B-iPS and MEF-derived iPS cells differentiated into lymphocytes in OP9 co-culture systems. Both efficiently differentiated into T-cell lineage that produced IFN-γ on T-cell receptor stimulation. However, iPS cells including B-iPS cells were relatively resistant to B-cell lineage differentiation. One of the reasons of the failure of B-cell lineage differentiation seemed due to a defect of *Pax5* expression in the differentiated cells. Therefore, current *in vitro* differentiation systems using iPS cells are sufficient for inducing T-cell but not B-cell lineage.

Keywords: B cell, development, differentiation, ES cells, haematopoietic cells, iPS cells, OP9, reprogramming, T cell

Introduction

Pluripotent stem cells are being used extensively in biomedical research as they are proving invaluable for an array of potential applications. Pluripotency can be induced in mouse and human somatic cells by forced expression of *OCT4* and *SOX2* with a combination of either *KLF4* and *MYC* or *NANOG* and *LIN28* (1–4), resulting in the formation of induced pluripotent stem (iPS) cells. Differentiation of iPS cells into various cell types belonging to the three germ layers has been demonstrated by the analysis of teratomas generated from mouse and human iPS cells. In addition, the pluripotency of iPS cells is further evidenced by the contribution of iPS cell-derived cells to development of various organs of chimeric mice developed from iPS cell-introduced blastocysts (5). Regarding *in vitro* generation of cells of mesodermal lineage from iPS cells, differentiation into cardiac myocytes and endothelial cells from mouse iPS cells has been recently reported (6–8). However, it remains to be determined whether fully differentiated and functional haematopoietic cells can be generated from iPS cells by direct differentiation *in vitro*. Senju *et al.* (9) recently reported that mouse iPS cells can differentiate into macrophages and dendritic cells. Lei *et al.* (10) recently reported that mouse iPS cells can differentiate into T cells. However, information regarding differentiation into B cells is limited.

Differentiation of iPS cells into haematopoietic cells including lymphoid lineage is being considered for establishing new therapeutic tools for treating some haematological or immunological disorders. To assess the lymphoid lineage differentiation from iPS cells, we used the *in vitro* OP9 co-culture system. It has previously been demonstrated that haematopoietic stem and embryonic stem (ES) cells can differentiate into both T and B cells in this system (11). We were able to induce differentiation of mouse embryonic fibroblast-derived iPS (MEF-iPS) cells and found that they could easily differentiate into T-cell lineage, but not B-cell lineage, using this system (described below). We hypothesized that

this disparity was dependent on the source of iPS cells. Therefore, we attempted to generate iPS cells from B cells instead, using the 'classical' retroviral transduction of four Yamanaka transcription factors (Oct4, Sox2, Klf4 and c-Myc) (3) and successfully generated iPS cells from murine splenic B-cell-derived iPS (B-iPS) cells. Here, we report the generation of B-iPS cells and their efficient and reproducible differentiation into T-cell lineage *in vitro*. In contrast, the results indicated that iPS cells are relatively resistant to differentiate into B-cell lineage *in vitro*.

Materials and methods

Mice and cell lines

Imprinting control region (ICR) mice and non-obese diabetic (NOD)-SCID mice were purchased from Japan CLEA, Inc. (Tokyo, Japan). C57BL/6-Ly5.1 mice were purchased from RIKEN Bioresource Centre (Ibaraki, Japan). All animal procedures were approved by the St Marianna University Animal Care Committee. The mouse iPS-MEF-Ng-38C-2 cell line (MEF-iPS), which was generated from MEFs by retroviral transduction of Oct4, Sox2, Klf4 and c-Myc (5), was kindly provided by Dr Yamanaka (Kyoto University). MEF-iPS cells were maintained in DMEM medium supplemented with 15% FCS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 10 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin (all from Invitrogen) containing 100× of recombinant human leukaemia inhibitory factor (LIF) supernatant (Wako, Tokyo, Japan) on feeder layers of irradiated MEF in 6-cm culture dishes. OP9 and OP9-DL1 cell lines were generous gifts from Dr Hiroshi Kawamoto [Research Center for Allergy and Immunology (RCAI), RIKEN, Yokohama, Japan] and were cultured as monolayers in OP9 media (α-MEM supplemented with 20% FCS, 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 U ml^{-1} penicillin, 100 μg ml^{-1} of streptomycin and 2.2 g l^{-1} sodium bicarbonate). The ES cell line (B6 Ly5.1 ES) was a generous gift from Dr Haruhiko Koseki (RCAI, RIKEN). ES cells were maintained in ES cell media DMEM high glucose supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 μM beta-mercaptoethanol, 15% FCS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} of streptomycin and recombinant human LIF supernatant on irradiated MEFs. MEFs were generated from embryos on day 14 as described previously (3).

Generation of iPS cells from peripheral B cells

pMXs vectors encoding Oct4, Sox2, Klf4 or c-Myc were established as described previously (3). Retroviruses were prepared as described previously (3, 5), and 8 μg ml^{-1} of polybrene (Sigma–Aldrich, St Louis, MO, USA) was added to the virus-containing supernatant. Murine splenic B cells were isolated with MACS beads (Miltenyi Biotech) as determined by $CD19^+$ expression (purity > 98%). The isolated $CD19^+$ cells were incubated in RPMI1640 medium supplemented with 10% FCS, 10 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol in the presence of 10 ng ml^{-1} IL-4 (Peprotech) and 1 μg ml^{-1} LPS (Sigma–Aldrich). After 24 h, the four reprogramming factors or Green fluorescent protein was introduced by retroviral transduction with centrifugation (780 × *g* for 60 min) and then incubated for 4 h in a 32°C, 5% CO_2 incubator and then incubated at 37°C. Viral transduction was performed twice over two consecutive days. Four days after the first transduction, RPMI medium was replaced with iPS cells medium. Twelve days after the first transduction, cells were plated onto irradiated MEF supernatant in iPS cells medium in 100-mm dishes. Seventeen days after the first transduction, iPS cell colonies were isolated in iPS cell lines.

Teratoma formation, histological examination and blastocyst injections

iPS cells (3×10^5) suspended in PBS containing 10% FCS were inoculated into testes of NOD-SCID mice. Four weeks after the injection, tumours were surgically dissected from the mice and fixed in 4% formaldehyde. The samples were embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin. For blastocyst injections, a controlled number of B-iPS cells was micro-injected into ICR blastocysts and transferred to pseudopregnant female mice.

OP9 co-cultures

Differentiation of iPS cells was induced with a withdrawal of LIF from the culture in a non-treated plastic dish. By day 5 of culture, embryonic body-like round-shaped spheres were formed. The spheres were disrupted with 0.25% trypsin (Gibco-BRL). The resultant single-cell suspensions were replated at a density of 6×10^5 cells per 100-mm non-treated dish containing fresh OP9 cells with the addition of Flt3 ligand (20 ng ml^{-1} for B-cell lineage or 5 ng ml^{-1} for T-cell lineage differentiation; R&D Systems). On day 8 of culture, loosely adherent haematopoietic cells were harvested by gentle pipetting. Every 6 days thereafter, non-adherent iPS cell-derived haematopoietic cells were collected by vigorous pipetting, filtered through a 70-μm nylon mesh and transferred onto fresh OP9 (for B-cell lineage culture) or OP9/DL1 (for T-cell lineage culture) monolayers in OP9 media. On day 8 of culture, another Flt3 ligand and exogenous IL-7 (5 ng ml^{-1}; R&D Systems) were added. Both cytokines were included at all subsequent passages.

Flow cytometry and antibodies

Flow cytometry was performed with an FACScalibur® or FC500® instrument and analyzed by CellQuestPro® or FlowJo® software. FITC-conjugated anti-CD3 (clone 145-2C11) and -CD45 (clone 30-F11); PE-conjugated anti-CD8 (clone 53-6.7), -CD19 (clone 1D3), -CD25 (clone 7D4), IFN-γ (clone XMG1.2), TCRγδ (clone GL3), Flk-1 (clone 89B3A5); allophycocyanin-conjugated anti-CD4 (clone GK1.5), CD11b (clone M1/70), CD44 (clone IM7), TCRβ (clone H57-597), biotin-conjugated anti-CD3 (clone 145-2C11) and CD19 (clone 1D3) mAbs were purchased from Biolegend. A PE-conjugated anti-FoxP3 (clone FJK-16s) was purchased from eBioscience. An FITC-conjugated TCR panel was purchased from BD Biosciences. For analysis, live cells were gated based on forward and side scatter as well as lack of propidium iodide uptake.

T-cell stimulation assay

Non-adherent cells from iPS-cells/OP9-DL1 co-cultures at day 21 were collected by vigorous pipetting and filtered through a 70-μm nylon mesh. Collected cells were pre-plated into a fresh dish and cultured for 1 h at 37°C in 5% CO_2 incubator to avoid contamination of MEFs. Then the collected cells were stimulated for 2 days with plate-bound anti-CD3 (1 μg ml^{-1}; clone 145-2C11) mAb in differentiation medium in the presence of IL-2 (1 ng ml^{-1}) and anti-CD28 (1 μg ml^{-1}; clone 37.51). Cells were subsequently cultured for 6 h in the presence of phorbol myristate acetate/ionomycin. Intracellular staining for IFN-γ was performed with Cytofix/Cytoperm® (BD Bioscience) according to the manufacturer's instructions. Cells were analyzed by flow cytometry.

Bisulphite genomic sequencing analysis

Genomic DNA was prepared by using QIAGEN DNA extraction kit (QIAGEN). Whole genomic DNA was bisulphited by MethylEasy™ Xceed (Human Genetic Signatures); follow the manufacture's protocol. The resultant DNA was amplified using AmpliTaq® DNA polymerase (Applied Biosystems) and primers specific for the Pax5 promoter region 5′-TGGTTGATAATTGTGTTAGTATAGGG-3′ and 5′-AAACCCAAAAAACAACAATACC-3′ under the following conditions: 95°C for 1 min, 35 cycles of 95°C for 30 s, 53°C for 90 s, 72°C for 60 s and 72°C for 10 min. PCR product was cloned into pCR-TOPO vector using TOPO® TA cloning kit (Invitrogen). At least five individual bacterial clones were analyzed. DNA sequencing was performed using ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

Gene rearrangement analysis

Genomic DNA was prepared by using QIAGEN DNA extraction kit (QIAGEN). PCR primers used for *Bcr* V-DJ and *Tcr* rearrangement analyses have been described previously (12, 13).

Reverse transcription–PCR

cDNA was generated with oligo dT primers and Superscript III (Invitrogen) from total RNA samples. Reverse transcription (RT)–PCR was performed with Amplitaq® (Applied Biosystems) for ES markers and lymphocyte differentiation markers. The primers used for ES cell markers analysis were described previously (3, 11). We used the following primers for development and differentiation analysis: 5′-ATGGAAGGGTTTTCCCTCACCGCC-3′ and 5′-GTCCACGCTCTGCAGCTCTGTGAA-3′ for *Pu.1*, 5′-TGCAGACATTCTAGCACTCTGG-3′ and 5′-ACATCTGCCTTCACGTCGAT-3′ for *Rag-1*, 5′-CCTGCCTCTCCTCCTCTTCT-3′ and 5′-CCCCTGGAGATGTCCTCATA-3′ for *Igα*, 5′-GATGCGGTGGAACACTTTCT-3′ and 5′-TAGTCTGGGTTGGGAACAGG-3′ for Cd3ε, 5′-CGCACTGACCACGAGCTTCAC-3′ and 5′-TCCAGGGACAGCACCTCATCTG-3′ for *E47*, 5′-AGCAACTGGACGCATGTATC-3′ and 5′-TCACCATCTCTGTAGTCAGG-3′ for *Il-7rα*, 5′-CAGAGCCTCCTCCCCCAACAG-3′ and 5′-GCTCAGAGGGGTGGGTAAGAT-3′ for *pTα*, 5′-TCCTCGGACCATCAGGACAG-3′ and 5′-CCTGTTGATGGAGCTGACGC-3′ for *Pax5*, 5′-ACTACCTCTGGAGCACAGCAGAA-3′ and 5′-ATAGGGCATGTCTGACAGGCACT-3′ for *Ikaros* and 5′-CCCTCCAACTGCAGTAGCTC-3′ and 5′-GCAAGGTCGGTGATTTTGTT-3′ for *Ebf1*. For *Oct4* detection, we used the following primers: 5′-CTGAGGGCCAGGCAGGAGCACGAG-3′ and 5′-CTGTAGGGAGGGCTTCGGGCACTT-3′ for total expression, 5′-TCCCTAGGTGAGCCGTCTTT-3′ and 5′-TTCATGTCCTGGGACTCCTC-3′ for internal expression, 5′-GTACAAAAAAGCAGGCTCCACC-3′ and 5′-GGTTCTCAATGCTAGTTCGCT-3′ for transcripts from *Oct4* vector. We also used the following primers: 5′-ACTTTTGTCCGAGACCGAGA-3′ and 5′-ATGTAGGTCTGCGAGCTGGT-3′ for *Sox2*, 5′-CAGCTTCATCCTCGTCTTCC-3′ and 5′-CGGGACTCAGTGTAGGGGTA-3′ for *Klf4*, 5′-GCCCAGTGAGGATATCTGGA-3′ and 5′-GAATCGGACGAGGTACAGGA-3′ for *c-Myc*. PCR products were separated by agarose gel electrophoresis and were visualized by ethidium bromide staining. All PCR products shown correspond to expected molecular sizes.

Results

Generation of B-iPS cells

To generate B-iPS cells, we first isolated peripheral $CD19^+$ B cells from mouse spleen. The isolated $CD19^+$ cells were also determined to be $CD24^+$, CD45R $(B220)^+$ and IgM^+ (data not shown). $CD19^+$ cells were then activated with IL-4 and LPS and transduced with four retroviruses encoding Oct4, Sox2, Klf4 or c-Myc. Within 17 days of culture after the transduction, we obtained ~25 iPS colonies from 4×10^6 $CD19^+$ cells in our first experiment and ~30 colonies from 1×10^7 $CD19^+$ cells in our second experiment.

The obtained B-iPS colonies were expandable in culture and exhibited morphology similar to mouse ES and MEF-iPS cells (Fig. 1A). B-iPS cells expressed ES cell marker genes including *Nanog*, *Ecat* and *Gdf* similar to MEF-iPS and ES cells (Fig. 1B). In contrast, B-iPS cells did not express B-cell-specific transcription factor *Pax5* (Fig. 1B). We confirmed the rearrangement of B-cell receptors in B-iPS cells. Eight of 12 B-iPS cell colonies revealed a VDJ3 band, similar to splenic $CD19^+$ cells (Fig. 1C, upper), whereas the remaining four colonies revealed a VDJ2 band (Fig. 1C, lower). These data indicate that the source of B-iPS cells was *Bcr* gene-rearranged B cells, which was inherited by B-iPS cells.

We further examined teratoma formation. We injected three B-iPS cell lines into the testes of NOD-SCID mice. Four weeks after inoculation, macroscopic teratomas were observed in all injected mice. Histological examination showed that teratomas contained cell types representing all three embryonic germ layers (Fig. 1D). Blastocyst injection of B-iPS cells resulted in generation of chimeric offspring mice (Fig. 1E). These data clearly demonstrate that iPS cells can be derived from mouse peripheral B cells by forcing expression of the four Yamanaka factors (3) without any additional factors.

B-cell lineage differentiation of iPS cells and ES cells

Schmitt *et al.* (11) previously showed that B cells can be differentiated from embryonic or haematopoietic stem cells by day 20 in OP9 co-culture system in the presence of Flt3L and IL-7. Thus, we anticipated that iPS cells could also be differentiated into B-cell lineage using the OP9 co-culture system with slight modification. In the first 5-day culture

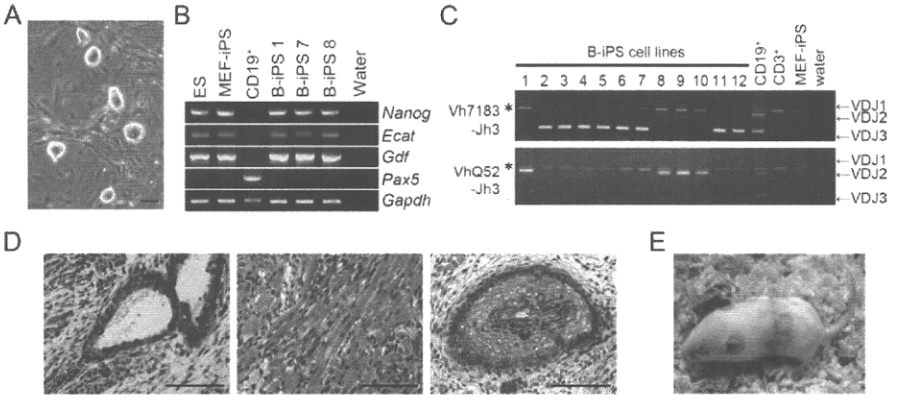

Fig. 1. Generation and characterization of B-iPS cells. (A) Morphology of iPS cells derived from mouse CD19$^+$ B cells. (B) ES cell marker expression of B-iPS cells. Transcripts from ES cells (R1), MEF-iPS cells, splenic CD19$^+$ B cells and three independently established B-iPS cell lines were analyzed by RT–PCR analysis. (C) Analysis of IgH V(D)J rearrangements in B-iPS cell lines. Genomic DNA was isolated from B-iPS cell line, splenic CD19$^+$ B cells, splenic CD3$^+$ T cells and MEF-iPS cells and analyzed by genomic PCR for the presence of IgH V(D)J gene rearrangements using primers as indicated. Asterisks indicate non-specific bands. (D) Histological analysis of teratoma from B-iPS cell line. Data of a B-iPS cell line 8 are shown. Thin-sectioned teratoma was stained by haematoxylin and eosin. Ciliated cell (endoderm, left), muscle fibre (mesoderm, centre) and dermal tissue (ectoderm, left) were shown. (E) Contribution of B-iPS cells to mouse embryonic development. B-iPS cells (line 8) were micro-injected in to blastocysts of ICR mice. Mice that have black- and white-coloured coat were born.

without LIF in a non-treated dish, embryonic body-like sphere formation was exhibited by B-iPS (Fig. 2A), MEF-iPS and ES cells (data not shown). These spheres contained mesoderm-like cells, which express Flk-1 (Fig. 2B). The spheres were then passed into OP9 feeder cells. By day 8 of the culture, cells similar to haematopoietic cells appeared (Fig. 2C). However, B-iPS and MEF-iPS cells only seldom showed B-cell lineage (CD19$^+$) differentiation (Fig. 3A), whereas ES cells and haematopoietic progenitor cells from foetal liver efficiently differentiated into a B-cell lineage (Fig. 3A and B). The MEF-derived iPS cell line (38C-2, established in Dr Yamanaka's laboratory, Kyoto University) occasionally expressed the B-cell marker CD19, but its frequency was very low compared with that of ES cells. We further tried the B-cell lineage differentiation from iPS cells by increasing the dose of cytokines, and by adding both vascular endothelial growth factor and bone morphogenetic protein-4, which were demonstrated to synergistically enhance lympho-haematopoietic cell generation (14), or LPS, which was shown to increase IgM$^+$-secreting B cells (15). However, these approaches did not enhance the differentiation of B-cell lineage in OP9 cells (data not shown). Therefore, even when B cells were used for the source of iPS cells, they are relatively resistant to differentiate into B-cell lineage with the current *in vitro* OP9 co-culture system. In addition, this co-culture did not induce differentiation of iPS cells into T-cell lineage that were traceable with the markers CD44, CD25, CD4 and CD8 (Fig. 3A).

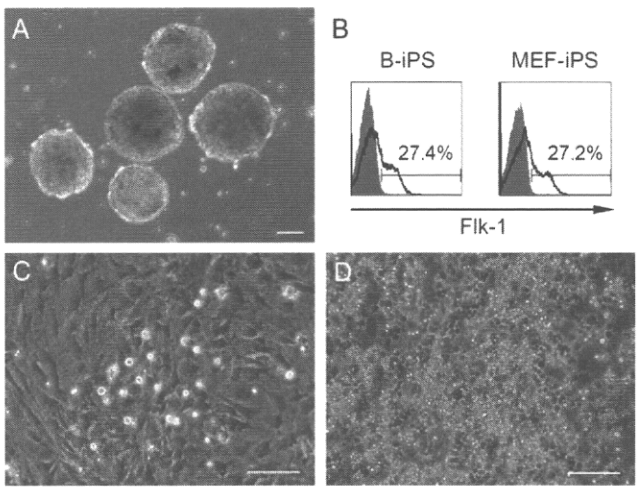

Fig. 2. Appearance of differentiating iPS cells. (A) Embryonic body-like spheres of B-iPS cells. B-iPS cells were cultured in differentiation media without LIF for 5 days. Scale bar: 100 μm. (B) Flow cytometry analysis of the embryonic body-like spheres. Single-cell suspension of the embryonic body-like sphere shown in (A) was examined for their expression of a mesoderm cell marker, Flk-1. (C) Hematopoietic cell formation. The single-cell suspension of embryonic body-like spheres was cultured on OP9 cells for another 3 days in the presence of Flt3 ligand. Scale bar: 100 μm. (D) Lymphocyte-like proliferation. At day 14, appearance of cells co-cultured with OP9-DL1 in the presence of Flt3 ligand and IL-7. Scale bar: 100 μm.

T-cell lineage differentiation of iPS cells

We further evaluated T-cell lineage differentiation of iPS cells *in vitro* using a co-culture system including OP9 cells expressing a Notch ligand, delta-like 1 (OP9-DL1), which has been shown to be essential for T-cell lineage differentiation (11). By way of embryonic body-like formation, B-iPS or MEF-iPS cells were co-cultured with OP9-DL1. By day 14, both iPS cell types turned into lymphocyte-like cells (Fig. 2D). On day 14, they gave rise to a population of cells expressing CD25 and/or CD44 and hence likely belonging to T-cell lineage resembling intrathymic differentiation (Fig. 4A). Rearrangement at the TCRβ locus (*Tcrβ*) is a hallmark of T-cell lineage commitment and is essential for progression of CD4/CD8 double-negative thymocytes to the double-positive stage, which occurs during normal αβT-cell development. To determine whether T cells derived from iPS cells co-cultured with OP9-DL1 cells undergo normal rearrangement

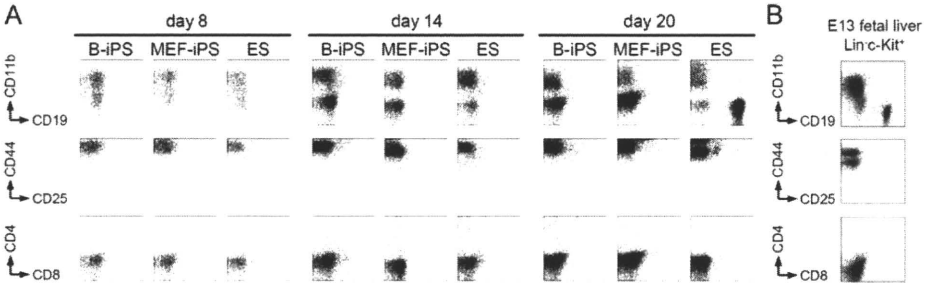

Fig. 3. Failure of B-cell lineage differentiation from iPS cells. (A) Flow cytometry analyses of B-iPS, MEF-iPS or ES cells differentiated with OP9 cells. On day 8, 14 and 20, expression of the indicated cell surface molecules on the differentiated cells were examined. (B) B-cell lineage differentiation of E13 fetal liver $Lin^-c\text{-}Kit^+$ cells on the OP9 monolayer. Flow cytometry was performed as in (A).

of the *Tcrβ* locus, we stained the differentiated cells collected on day 30 with various antibodies against TCRβ chain. The repertoire of TCRVβ in the generated T cells derived from B-iPS cells contained diversities as did those from MEF-iPS cells (Fig. 4B and data not shown). These results were confirmed by genomic PCR (Fig. 4C). Taken together, these data indicate that the iPS cell-derived T cells developed in the OP9-DL1 co-culture system have the potential to generate a diverse TCR repertoire.

During normal thymocyte development, T cells bearing both TCRαβ and TCRγδ develop in the thymus. To determine whether both populations of T cells also develop from iPS cells co-cultured with OP9-DL1 cells *in vitro*, we analyzed iPS cell-derived T cells for surface expression of TCRαβ and TCRγδ. These results showed that both populations of T cells developed from iPS cells in the co-culture model (data of B-iPS cell are shown; Fig. 4D).

iPS cell-derived T cells collected on day 20 and thereafter contained cells that were both CD4/CD8 double-positive and $CD8^+$ cells (Fig. 4A). To determine whether the TCRs expressed on these T cells were indeed functional, we stimulated the cells for 3 days in the plate-bound anti-CD3 antibody. Certain populations of the iPS cell-derived T cells produced IFN-γ in response to the TCR stimulation (Fig. 4E, gated on CD8). Furthermore, we added transforming growth factor-β to the culture of iPS cell-derived T cells in the presence of TCR stimulation. The addition of transforming growth factor-β enhanced the population of $FoxP3^+$ cells (Fig. 4F), which is the hallmark of regulatory T cells, as shown in naive T cells derived from normal adult lymphoid tissue (16). These data suggest that the iPS cell-derived T cells generated in this co-culture can respond to stimulation via TCR or cytokine receptors to a certain extent similar to naive T cells.

Analysis of gene expression in differentiating iPS cells

To elucidate the differentiation process of B-iPS cells at the molecular level, we assessed the expression of developmentally regulated genes by RT–PCR analysis. We analyzed the transcripts from whole cells of differentiating B-iPS, MEF-iPS and ES cells at day 20 of culture. A zinc finger transcription factor, *Ikaros*, and an Ets protein, *Pu.1*, both of which are known to be critical in regulation of haematopoiesis, were distinctly expressed in differentiating iPS cells co-cultured with either OP9 or OP9-DL1 (Fig. 5A and B, respectively). We also analyzed expression of the gene encoding the IL-7 receptor (*Il7r*), which is required for the survival and proliferation of lymphocyte progenitors. We found the *Il7r* transcripts in the differentiating cells from both conditions of T-and B-cell lineage differentiation (Fig. 5). Differentiating cells co-cultured with OP9-DL1 cells expressed substantial levels of Cd3, *Rag1* and *pTα*, which are essential for T-cell lineage development and were observed in normal thymocytes (Fig. 5B). These gene expression data are in agreement with the apparently normal development of T-cell lineage from iPS cells in OP9-DL1 co-culture (Fig. 4). In contrast, iPS cell-derived, but not bone marrow- or ES cell-derived, differentiating cells co-cultured with OP9 cells failed to express *Pax5*, which is critical for the development of B-cell lineage (Fig. 5A). Accordingly, transcripts encoding for Rag1, which is required for the B cell receptor (BCR) rearrangement, and for Igα, which is part of the pre-BCR complex, were not present in the cells differentiated from iPS cells co-cultured with OP9 cells (Fig. 5A). These data are in agreement with the fact that it was difficult to induce B-cell lineage differentiation using this co-culture system (Fig. 3). These data also suggest that the induction of T-cell, but not B-cell, lineage-specific gene expression in differentiating iPS cells was appropriately co-ordinated in these culture conditions. The immature regulated gene expression in iPS cells co-cultured with OP9 cells seemed to induce incomplete differentiation into B-cell lineage.

CpG methylation status of Pax5 promoter

Transcriptional silencing caused by DNA methylation of Pax5 promoter was observed in terminally differentiated B-cell lines (17). It was anticipated that such a CpG methylation in iPS cells caused the failure of *Pax5* expression. Thus, we analyzed the CpG sites in TATA-containing upstream promoter of *Pax5* in iPS cells. Bisulphite genomic sequencing analysis revealed that the *Pax5* promoter region in B-iPS and MEF-iPS cells were largely unmethylated as in splenic $CD19^+$ cells and ES cells (Fig. 6). It suggests that the failure of *Pax5* expression in iPS cells was not caused by the epigenetic modification of the *Pax5* gene.

Gene expression analysis in early stage of differentiation

To obtain insights about the failures of the B-cell lineage development from iPS cells, we further analyzed the B-lymphopoiesis in earlier stage of differentiation. On day 8 of

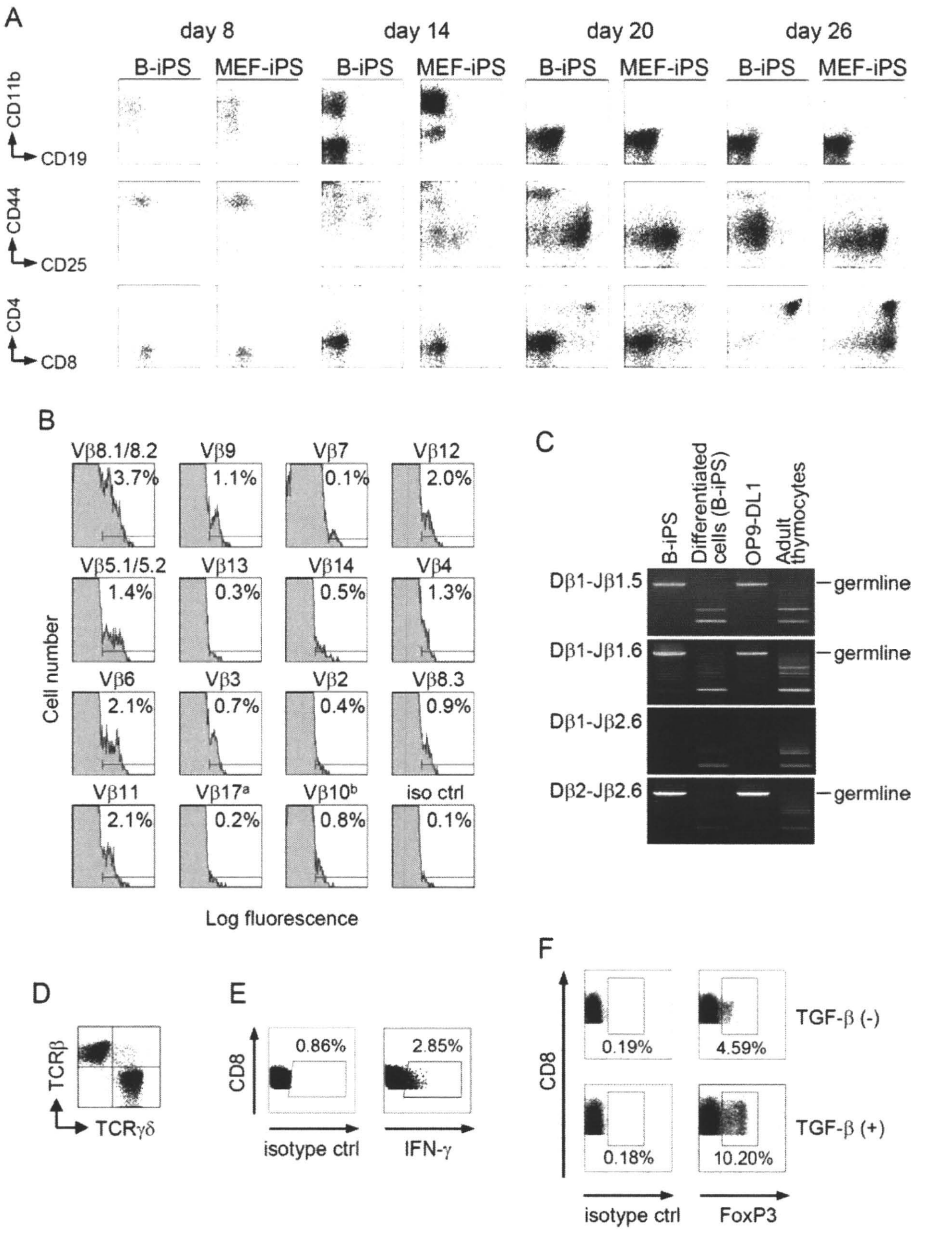

Fig. 4. Efficient T-cell lineage differentiation from iPS cells. (A) Flow cytometry analyses of iPS cells differentiated with OP9-DL1 cells. On day 8, 14, 20 and 26, expression of the indicated cell surface molecules on the differentiated cells were examined. (B) TCRVβ chain repertoire was analyzed with flow cytometry. The B-iPS cells differentiated with OP9-DL1 were examined at day 30 of culture. $CD3^+$ cells were gated. (C) Gene rearrangement analysis of *Tcr* locus. Genomic DNA was isolated from OP9-DL1, B-iPS cells (undifferentiated), B-iPS cells co-cultured on OP9-DL1 and mouse adult thymocytes as a control. *Tcr* gene rearrangement was analyzed by PCR. (D) αβT-cell and γδT-cell generation from B-iPS cells (day 30). $CD3^+$ cells were gated. (E) Intracellular analysis of IFN-γ secretion. Day 23 of the OP9-DL1 co-culture, generated cells from B-iPS cells were collected by vigorous pipetting through a 70-μm nylon mesh. Harvested cells were stimulated as described in Methods, and Golgi stop solution was put into the culture 6 h before the analysis. (F) Induction of $FoxP3^+$ regulatory T cells from B-iPS cell-derived T cells. Day 22 of the OP9-DL1 co-culture, differentiated cells were collected and put into the anti-CD3 mAb-coated well and cultured for another 2 days with 2 ng ml^{-1} of IL-2, with/without 5 ng ml^{-1} of transforming growth factor-β1. Intracellular FoxP3 expression was analyzed. $CD4^-CD8^+$ cells were shown.

the culture, losely attached cells were harvested and then c-kit^+ cells were magnetically sorted. Most of them expressed CD34 (data not shown), suggesting that these cells correspond to haematopoietic progenitor cells.

Ebf1 is one of the principle determinants of the B-cell fate (19, 20), and its promoter is activated by one of the E2A splicing variants, E47 (21). *E47* was detected in the differentiated cells derived from B-iPS, MEF-iPS or ES cells as well as bone marrow $linage^-$ c-kit^+ cells (Fig. 7A). mRNAs for Id proteins, which are known to bind to the E-proteins and prevent them from binding to DNA, as well as *Ebf1*, were also detected in all the cell types tested. On the other hand, *Pax5* expression was not detected in iPS cell-derived haematopoietic progenitor cells (Fig. 7A). In this experiment, the Pax5 expression was not

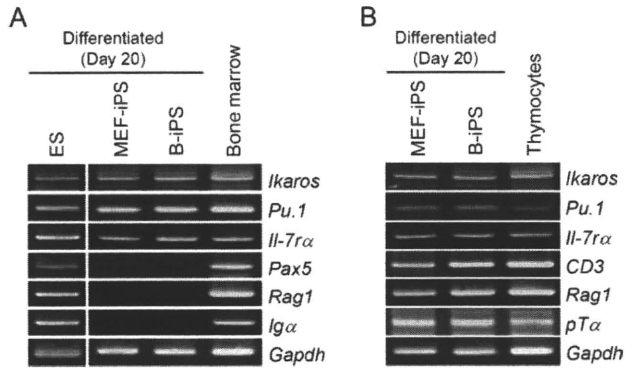

Fig. 5. Expression analysis of B- or T-cell lineage-correlated genes. B-iPS, MEF-iPS or ES cells were co-cultured on OP9 (A) or OP9-DL1 (B) for 20 days and transcripts analyzed from whole cells of differentiating B-iPS, MEF-iPS and ES cells. Mouse adult bone marrow (A) or thymocytes (B) were used as a control. The transcripts were analyzed by RT-PCR. Amount of transcripts between each samples were normalized by glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

detected in ES cell-derived cells either (Fig. 7A), but it clearly appeared in a later stage (see Fig. 5). Therefore, it seemed that, in the differentiated cells from iPS cells, the Pax5 expression was inhibited throughout the differentiation process.

Expression of the four Yamanaka factors in haematopoietic progenitors from iPS cells

We finally analyzed the expression of *Oct4*, *Sox2*, *Klf4* and *c-Myc* in the differentiated cells (Fig. 7B). *Klf4* and *c-Myc* expression were detected in the differentiated cells derived from B-iPS, MEF-iPS or ES cells as well as bone marrow linage$^-$ c-kit$^+$ cells. *Sox2* transcript was not detected in those cells. Importantly, *Oct4* transcripts were detected in B-iPS or MEF-iPS cell-derived haematopoietic progenitors and also slightly in the cells derived from ES cells. Detailed analysis using retrovirus vector-specific primers revealed that the *Oct4* transcripts in the cells derived from MEF-iPS or B-iPS cells were retrovirally transduced ones, but those in the cells derived from ES cells were endogenous (Fig. 7B). The ectopic *Oct4* expression seen in the cells differentiated from iPS cells may regulate the Pax5 expression and also B-cell lineage differentiation.

Discussion

In vitro culture methods are invaluable for defining specific cellular and genetic mechanisms that mediate lymphocyte development. It is widely known that ES cells can be differentiated into most blood cell lineages *in vitro* (22). The most commonly used method for inducing differentiation is the OP9 co-culture system. The OP9 cell line was established from calvariae of newborn *op/op* mice, which lack functional macrophage colony-stimulating factor (M-CSF) (23). OP9 cells enhance haematopoietic development by providing a supportive microenvironment for differentiation. The absence of M-CSF inhibits survival of monocytes and macrophages, which tend to outgrow other lineages in systems using wild-type stroma. Using this co-culture system, it has been well documented that ES cells can yield erythroid, myeloid and B-cell lineage cells (reviewed in ref. 22). However, it has been relatively difficult to generate T cells from unmanipulated ES cell-derived haematopoietic progenitor cells. Schmitt *et al.* (11) demonstrated an efficient induction of T cells from ES cells using OP9-DL1 cells that ectopically expressed the Notch ligand Delta-like 1. They showed that ES cells cultured with OP9-DL1 cells differentiated into haematopoietic cells, committed to T-cell lineage, underwent stage-specific proliferation and matured into CD4$^-$ and/or CD8$^+$ T cells *in vitro*. On the other hand, ES cells cultured on control OP9 cells differentiated into B-cell lineage as had been reported (11).

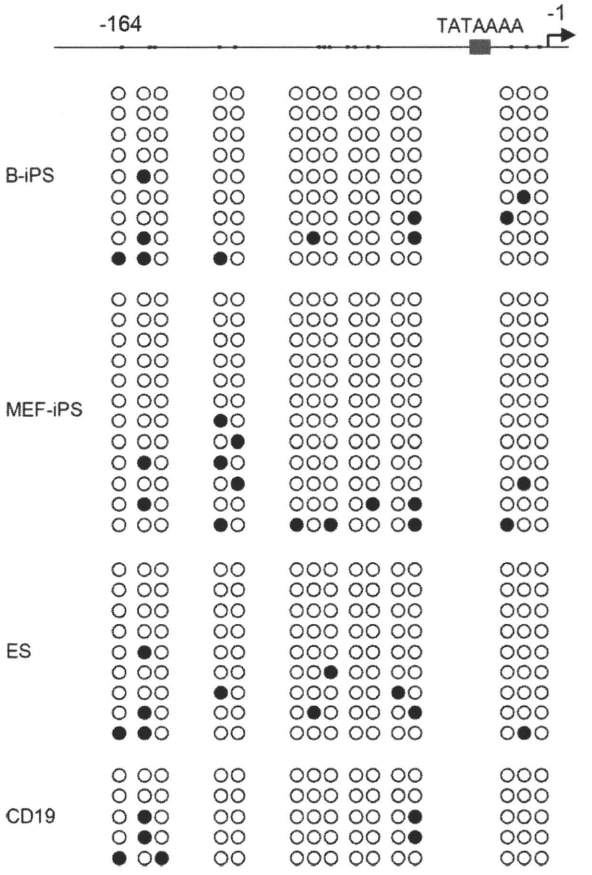

Fig. 6. Defect of *Pax5* expression was not due to CpG methylation of *Pax5* promoter. CpG methylation status of the *Pax5* promoter region containing 200 bp upstream from the transcription initiation site was analyzed by bisulphite genomic sequencing. Numbering at the top corresponds to the position relative to the published transcription initiation site (arrow) (18), and the filled small box indicates the position of the CpG site. Each row of circles represents a single cloned allele, and each circle represents a single CpG site (open circle, non-methylated cytosine; filled circle, methylated cytosine).

Using the above-mentioned OP9 co-culture system, in this study, we showed that iPS cells are competent to differentiate into T-cell lineage but are relatively resistant to differentiation into B-cell lineage *in vitro*. We used both MEF- and B-cell-derived iPS cells but failed to demonstrate reproducible B-cell lineage development *in vitro*. Analyses of gene expression data indicated that iPS cells are defective in expressing genes required for B cell, especially Pax5 throughout the differentiation process (Fig. 7), suggesting

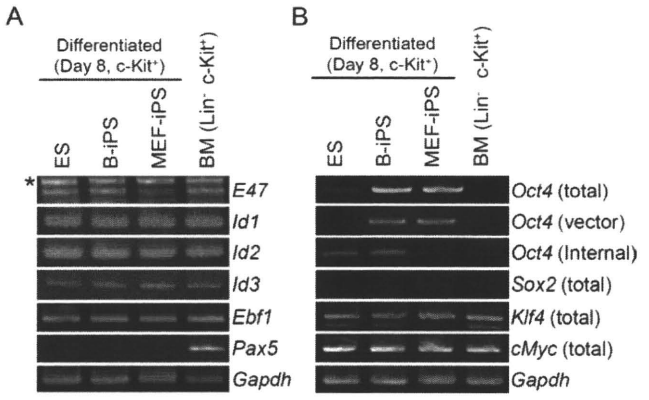

Fig. 7. Expression analysis in early stage of differentiation. B-iPS, MEF-iPS or ES cells were co-cultured on OP9 for 8 days, and c-kit$^+$ cells were harvested. Bone marrow lineage$^-$ c-kit$^+$ cells were used as a control. Transcripts for B-cell lineage development (A) and four Yamanaka factors (B) were analyzed by RT-PCR. Amount of transcripts between each samples was normalized by *Gapdh*. Asterisks indicate non-specific bands.

that there is no sufficient reprogramming in the genetic region of iPS cells that is required for B-cell development. However, in the case of Pax5 promoter region, it seemed unlikely that an aberrant CpG methylation induced some epigenetic modifications, which are responsible for the loss of Pax5 expression (Fig. 6). Alternatively, some transcriptional or translational changes in iPS cells may affect gene expression required for B-cell development but not for T-cell development. In the absence of Pax5, B-cell development is arrested at the early pro-B-cell stage of development (24), and *Ebf1* expression appears important to activate the B-cell lineage programme (25). Therefore, we tried to rescue the B-cell lineage differentiation by enforced expression of Pax5 or Ebf1 in differentiated cells from iPS cells by using retroviral vectors encoding *Ebf1* or *PAX5* that have been reported to induce the B-lymphopoiesis (kindly provided by Dr Ikawa, ref. 12). However, these attempts did not induce the B-cell lineage differentiation from iPS cells (data not shown), suggesting an existence of some active inhibitor(s) for B-cell differentiation in the differentiated cells from iPS cells. In this context, the detection of *Oct4* (including transduced one) (Fig. 7) may explain the resistance of B-cell lineage differentiation as it has been reported that the amount of Oct4 affects the development and differentiation of ES cells (26).

Although no precise explanation has been offered to account for the difficulty of B-cell lineage differentiation from iPS cells, similar differences in lymphocyte development potential between human embryonic and umbilical cord blood-derived progenitor cells have been reported (27). Using a co-culture system similar to those we employed, the authors found that ES cells could be used to efficiently create functional NK cells, whereas T- and B-cell development was much more limited. In contrast, umbilical cord blood stem cells routinely generated NK, T cells and B cells in the co-culture system. Accordingly, the authors found that ES cell-derived, but not umbilical cord blood-derived, haematopoietic progenitor cells constitutively expressed some transcriptional factors, including the ID family genes (27), which promote NK cell development and repress both T-cell and B-cell development, by inhibiting the E-protein family of basic helix-loop-helix transcription factors such as E2A (28–30). In the B-cell lineage differentiation form iPS cells, similar transcriptional mechanisms may operate which repress gene expressions required for the B-cell development. In fact, the transcripts of ID family genes were detected in haematopoietic progenitor cells from iPS cells (Fig. 7A). Further studies are needed to clarify these issues.

In this study, we successfully reprogrammed peripheral B cells into iPS cells. Most published protocols are optimized to reprogramme adherent cells, such as fibroblasts and keratinocytes from skin or hair (31–35). However, using these sources requires time-consuming skin biopsies and expansion *in vitro* for several passages, which make the method relatively cumbersome, particularly when generating patient-specific iPS cells. In this sense, it is desirable to reprogram blood cells that are easily accessible and less exposed to environmental mutagens.

Recently, iPS cell lines were derived from bone marrow progenitor cells obtained from a mouse whose haematopoiesis was reconstituted from a single congenic haematopoietic stem cell, providing evidence that mouse haematopoietic cells can be reprogrammed to pluripotency (36). Derivation of iPS cells from post-natal human blood cells has also been reported. One study indicated that granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood CD34$^+$ cells from a healthy donor were reprogrammed to iPS cells (37). More recently, it was reported that CD34$^+$ cells from human cord blood and adult bone marrow from healthy donors could be reprogrammed to iPS cells without pre-treatment, like G-CSF mobilization (38). Moreover, in this report, several iPS cell lines were established from peripheral blood CD34$^+$ cells containing the JAK2-V617F mutation that is commonly found in haematopoietic progenitor cells of adult patients with myeloproliferative disorders (38). However, these reports all employed haematopoietic progenitor or stem cells as the source of iPS cells, which usually involve some complicated procedures.

Lymphocytes have been used for the source of iPS cells previously. The first report indicating a reprogramming of mouse B lymphocytes to pluripotency proved that terminally differentiated somatic cells are receptive to being reprogrammed to iPS cells (39). In this report, it was indicated that only pro- and pre-B-lymphocytes were reprogrammed with the four factors, whereas mature B lymphocytes were reprogrammed by the additional over-expression of C/EBPα or specific knockdown of the Pax5 transcription factor (39). Eminli *et al.* (40) reported that terminally differentiated B and T lymphocytes could be reprogrammed with over-expression of the four Yamanaka factors, but the efficiency was quite low, with levels up to 300 times lower than haematopoietic stem and progenitor cells. In these studies, 'secondary' iPS cells were derived from primary B or T lymphocytes of adult spleen, bone marrow, lymph nodes or embryonic liver of mice engineered to carry doxycycline-inducible *Oct4*, *Sox2*, *Klf4* and *Myc* retroviruses in every tissue (39, 40). Similarly, Hong *et al.* (41) recently reported that murine splenic T lymphocytes could be reprogrammed to iPS cells although only when *p53*-null, but not wild-type, mice were used as the

source of lymphocytes. Therefore, it has been concluded that mature B and T lymphocytes are resistant to reprogramming with the four Yamanaka factors when no additional modification is made. Although the efficacy of B-iPS cells generation was quite low in our study, the fact that we generated iPS cells from peripheral B cells using the 'classical' method (3) might expedite new studies in which human peripheral B cells are examined for use as a source for iPS cells generation, largely because they are much more accessible.

Gaining a better understanding of differentiation of T or B lymphocytes from pluripotent stem cells *in vitro* can potentially guide development of new therapeutic strategies against some immunodeficiency diseases, infectious diseases or cancers in the field of regenerative medicine. Our present study highlights an important issue that applies to the derivation of virtually all cell types from iPS cells and not just B lymphocytes with *in vitro* co-culture systems. *In vitro* culture and differentiation of iPS cells leads to complex conditions and results in a heterogeneous mixture of progenitor cells that are stimulated by stromal cells and multiple soluble proteins that affect iPS cells lineage differentiation and development. Additional cellular and molecular studies of iPS cells are necessary to reveal unknown experimental and clinical potentials of these cells, particularly in terms of their tendency in lineage commitment.

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The authors declare no competing financial interest.

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Notch ligands transduce different magnitudes of signaling critical for determination of T-cell fate

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Notch signaling mediated by Delta-like (Dll) 4 is essential and sufficient for T-cell development *in vivo*. Stromal cells expressing Dll4 or Dll1, but not Jagged1, support T lymphopoiesis *in vitro*, but the molecular basis of this functional divergence among Notch ligands remains to be clarified. To examine this, we constructed chimeric variants composed of Dll4 and Jagged1. The intracellular regions were necessary, but interchangeable, for signal induction, and the extracellular regions determined the unique characteristics of the ligands. While Jagged1 induced minimal Notch signaling, Jagged2 elicited substantial levels of Hes1 transcripts and promoted T lymphopoiesis *in vitro*. Dll4 and Jagged2 showed a quantitative advantage when bound to fringe-modified Notch; this was not due to the Delta-Serrate-Lag2 domain, an extracellular region essential for interaction with Notch. These results suggest that different Notch ligands possess distinct potentials for the induction of Notch signaling through unique interactions of their extracellular regions with fringe-modified Notch. Furthermore, the magnitude of Notch signaling induced is critical for the determination of T-cell fate.

Key words: Notch ligand · Notch signaling · T-cell development

Supporting Information available online

Introduction

Notch receptors and their ligands, which are highly conserved from invertebrates to mammals, are expressed as transmembrane proteins and regulate cell-fate decisions in many tissues and organisms [1]. Such signals are transmitted between cells in direct contact with each other by the specific binding of Notch (Notch1–4 in mammals) and its ligands, Delta-like (Dll) or Jagged family members (Dll1, Dll4, Jagged1 and Jagged2 in mammals). These interactions result in proteolysis of Notch and translocation of the intracellular region of Notch into the nucleus, with the DNA-binding protein RBP-J acting as an ortholog of Drosophila Su(H). This translocation is an essential part of the signal transduction process and induces the transcriptional activation of the HES/E(spl) family. Notch signaling has been shown to affect a broad range of cellular functions, including proliferation, apoptosis and developmental lineage choices [2–4]. In the hematopoietic system, Notch signaling plays a critical role in promoting T-cell development.

T cells primarily develop in the thymus after migration of hematopoietic progenitor cells (HPC) from fetal liver (FL) or bone marrow, and undergo TCR gene rearrangement to generate a widely diverse repertoire. Studies using conditional gene targeting for Notch1 or RBP-J, and enforced expression of the active form of Notch1 in HPC, indicated that Notch1- and RBP-J-mediated signaling are both essential and sufficient for

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T-cell specification at the developmental branch point of T *versus* B cells [5–10]. Recently, using gene deletion, we demonstrated that in the thymic environment, Dll4, but not Dll1, on epithelial cells is indispensable for the induction of Notch signaling in thymic immigrants [10, 11]. Thus, it is obvious that Notch signaling induced by the interaction of Notch1 on the immigrants and Dll4 on the epithelial cells is critical for T-cell development in the thymus.

In contrast to Dll1 and Dll4, Jagged1 failed to induce sufficient Notch signaling in HPC for T lymphopoiesis *in vitro* [12, 13]. Abundant expression of Jagged1 was detected on osteoblasts, thus providing a hematopoietic niche in bone marrow [14]. These observations are consistent with the fact that T lymphopoiesis is mainly found in the thymic environment *in vivo*, not in bone marrow, and might explain the different potential of the primary lymphoid organs to support T-cell development. Several previous reports showed that the over-expression of Lunatic fringe (Lfng), a glycosyltransferase, in CD4/CD8-double positive (DP) thymocytes increased their responsiveness to Notch ligand (NotchL) expressed on the thymic epithelium and made them superior competitors for the ligand, while Lfng-deficient HPC did not efficiently develop into T-lineage cells when competing for ligands in the thymus. These findings suggested that Dll family members, which might require fringe-mediated modification of Notch in order to induce substantial signaling in recipient cells, may act as NotchL in the thymus, and that Lfng physiologically functions to modify the signaling capability of Notch in HPC [15–18]. Dll4 is essential for T-cell development in the thymus, but it remains unclear how Dll4 and Jagged1 transduce different signals, which seem to be critical for the determination of T-cell fate, and how Lfng affects their function. To address this issue, we established various transfectants expressing NotchL and chimeric molecules of Dll4, Jagged1 and Jagged2, and examined their ability to induce Notch signaling and support T lymphopoiesis *in vitro*. These experiments clearly show that functional differences among NotchL are due to the magnitude of Notch signaling induced and that this magnitude is critical for T-cell induction. The amount of Notch signaling is determined by the interaction between fringe-modified Notch and the extracellular regions of the ligands, which are functionally separated into the Delta-Serrate-Lag2 domain and other parts.

Results

Expression and signaling of chimeric NotchL

We, and other groups, reported that Dll1 and Dll4 on bone marrow-derived stromal cells supported T lymphopoiesis from HPC *in vitro*, but Jagged1 did not [9–13]. However, it still remained to be clarified why Notch signaling through different NotchL did not give rise to a similar phenotype. To address this issue, we performed further characterization of the Notch signaling induced by various NotchL. Dll1/4 and Jagged1 differ structurally in their intracellular and extracellular regions except for the Delta-Serrate-Lag2 (DSL) domain that is essential for binding to Notch and is well conserved in all NotchL. To analyze which of the intracellular or extracellular regions contribute to the functional divergence of NotchL, we generated deletion mutants lacking the intracellular region of Dll4 (D4d) or Jagged1 (J1d), and chimeric molecules such as D4d-J1 (the extracellular region of Dll4 with the transmembrane and intracellular regions of Jagged1) and J1d-D4 (the extracellular and transmembrane regions of Jagged1 with the intracellular region of Dll4) (Fig. 1A). Using these mutants we established transfectants in a fibroblast cell line derived from NIH-3T3, which supports B lymphopoiesis *in vitro*. Expression levels of NotchL could be monitored by the simultaneous expression of GFP, as these were derived from a bicistronic mRNA (Fig. 1B). Transfectants were examined by cell surface staining with mAb recognizing the extracellular region of the corresponding NotchL [19]. Flow cytometric analysis showed that the chimeras react to the corresponding antibodies even if the NotchL variant lacks the intracellular region (Fig. 1B). Although it was difficult to compare the relative surface expression levels by using ligand-specific mAb, we determined that D4d-J1 was highly expressed on the surface in comparison to Dll4 (Fig. 1B).

To verify the function of the chimeric NotchL, the transfectants expressing these NotchL were examined by inducing Notch signaling in newly generated Notch1-transfected NIH-3T3 (N1/3T3) using transient reporter assays with a reporter plasmid containing multiple repeats of an RBP-J-binding sequence [20] (Fig. 1C). The results showed that Notch signaling is transmitted through WT and chimeric NotchL only if they contain an intracellular region. These findings indicated that the intracellular regions of NotchL are essential for activation of Notch signaling as shown previously [21], but that the intracellular regions of Dll4 and Jagged1 are interchangeable. However, D4d-J1 might be less functional than intact Dll4, since D4d-J1 is much more highly expressed on the surface than Dll4 but induced the same level of signaling as Dll4 (Fig. 1C). Mock transfectants induced no signaling, suggesting that the dull staining with anti-Jagged1 mAb (Fig. 1B, Mock with a-Jag1) was due to nonspecific binding to NIH-3T3.

Extracellular region of NotchL determines its ability to induce T-cell development

Next, we examined whether Notch signaling *via* chimeric NotchL molecules possesses the potential to promote T lymphopoiesis (Fig. 2A). For this purpose, lineage markers-negative, c-kit-positive FL (LK-FL) cells, as a source of HPC, were cultured for 1 wk on transfectants expressing the various NotchL, in the presence of IL-7. Live cells were then harvested and analyzed for the expression of Thy-1/CD19 and CD44/CD25 after gating out NK1.1-, CD11b- and Gr1-positive cells. The result showed that LK-FL cells cultured on stromal cell transfectants expressing Dll4 and D4d-J1 developed into Thy-1$^+$CD25$^+$ T-lineage cells as

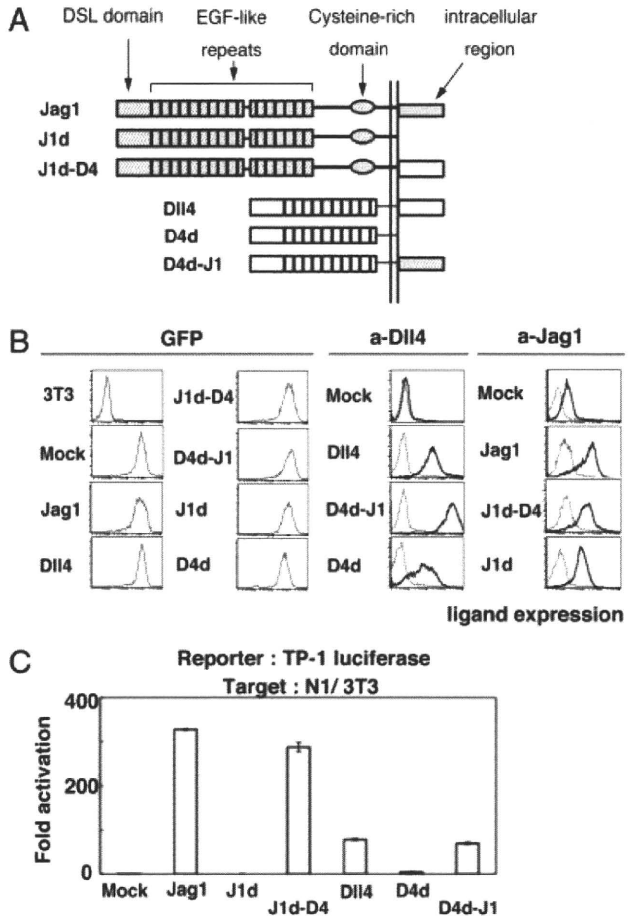

Figure 1. NotchL variants expressed on NIH-3T3 are capable of Notch signal transduction. (A) Schematic structure of the NotchL variants. Dll4 and Jagged1 are intact NotchL. D4d and J1d are shown as the deletion variants without the intracellular region of Dll4 and Jagged1, respectively. D4d-J1 and J1d-D4 are represented as the chimeric molecules of the ligands. D4d-J1 is constructed to contain the extracellular region of Dll4 and the intracellular region of Jagged1. J1d-D4 is composed of the extracellular region of Jagged1 and the intracellular region of Dll4. The DSL domain, EGF-like repeats and intracellular region of Jagged1 are indicated by light-gray rectangles, and those of Dll4 are represented by open rectangles. The circles indicate the cysteine-rich domain of Jagged family members. The transmembrane region of Jagged1 is indicated by a bold line and that of Dll4 by a thin line. (B) Flow cytometric analysis of NotchL (Jagged1 and Dll4), their variants and mock transfectants in NIH-3T3. All transfectants were established by the infection of retroviruses encoding NotchL as described in the *Materials and methods*. These cells were checked for GFP expression and stained with biotinylated anti-Jagged1 or Dll4 mAb and streptavidin-PE, and analyzed by flow cytometry. The thin line indicates the profile with biotinylated control hamster IgG as a negative control. (C) Chimeric variants with Dll4 and Jagged1 transduce Notch signaling. A stable transfectant of murine Notch1 (N1/3T3) was transiently transfected with a TP1-luciferase reporter plasmid, pGa981-6, and a pRL-TK plasmid for internal control. Cells were harvested at 24 h after transfection, and co-cultured for an additional 40 h with transfectants expressing NotchL. After background subtraction of endogenous Notch signaling (measured in Empty/3T3 or Lfng/3T3 without endogenous Notch1), the relative induction of luciferase activity in each sample (mean+SD, $n = 3$) was calculated and described as fold activation against the control (value from the culture with Mock transfectant not expressing any NotchL). Data are representative of five independent experiments.

efficiently as those cultured on the stromal cells expressing Dll1. Similar results were obtained with bone marrow-derived HPC (data not shown). On the other hand, Jagged1 and J1d-D4 did not promote T-cell development but rather allowed the appearance of B cells, as did mock transfectants. When cultured for additional time with PA6-derived transfectants expressing Dll4, D4-J1 or Dll1, the Thy-1$^+$CD25$^+$ T-lineage cells further developed into DP cells (Fig. 2B). These results indicated that the extracellular region, but not the intracellular region, of Dll4 determines the specificity of the ligand for T-cell induction.

Jagged2 promotes T-cell development

It was obvious that Jagged1 and some variants with a Jagged1-derived extracellular region did not promote T-cell development, in contrast to Dll4 and Dll1. To identify the key difference among NotchL, we next focused on another Jagged family molecule, Jagged2, which has extracellular domain structures analogous to Jagged1. We established transfectants of Jagged2 with expression levels comparable to that of Jagged1 as monitored by GFP expression (Fig. 3A, +); surface expression was confirmed using mAb specific for the extracellular region of Jagged2 [19] or HRJ1-5, which recognizes the extracellular regions of both Jagged1 and Jagged2 (HY, unpublished results) (Fig. 3A). The expression of Jagged1 on the surface of its transfectant was also detected by staining with HRJ1-5 (Fig. 3A, +). Thus comparing the surface expression of Jagged1 and Jagged2, it appeared that the levels were comparable.

The Jagged2 transfectant transduced Notch signaling greater than that induced by Jagged1 (Fig. 3B, +). Surprisingly, in contrast to Jagged1, the Jagged2 transfectant supported substantial T lymphopoiesis *in vitro* (Fig. 3C, Positive (+)), although its efficiency was lower than that of transfectants expressing Dll1 or Dll4 (Fig. 2). To confirm its function, we established additional transfectants expressing the ligands at higher levels (Fig. 3A, ++). The Jagged2 (++) transfectant could transduce greater signaling (Fig. 3B, ++), and, remarkably, supported T lymphopoiesis *in vitro* (Fig. 3C, High positive (++)). Moreover, high levels of Jagged2 expression on PA6 also supported differentiation to the DP stage as well as Dll4 and Dll1 (Fig. 2B), in contrast to Jagged1 (Fig. 3D). This demonstrated clearly that not only the Dll family molecules but also the Jagged family molecules can promote T-cell development without a thymic environment.

The magnitude of Notch signaling is regulated by Lfng and determines T- *versus* B-cell fate

The magnitude of the signal induced by various NotchL-expressing stromal cells on NIH-3T3 cells transfected with Notch1 was not consistent with their ability to induce T lymphopoiesis. As the extracellular region of NotchL is critical for T-cell induction and as Lfng functions as a glycosyltransferase and acts on the EGF repeats of the Notch receptor, we analyzed the role of

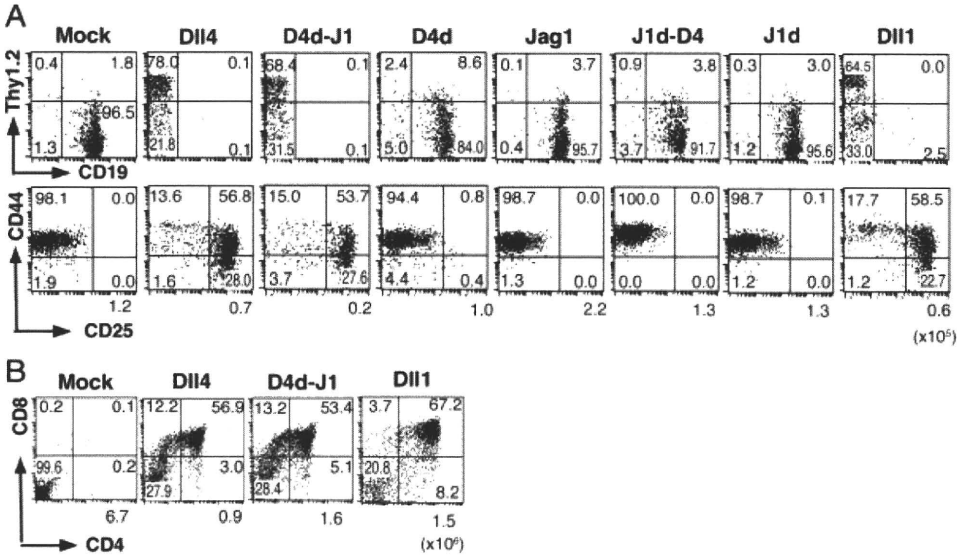

Figure 2. Induction of T-lineage cells by NotchL variants *in vitro*. (A) The extracellular regions of NotchL determine their ability to support T lymphopoiesis. LK-FL cells were cultured on monolayers of various transfectants of NotchL in the presence of IL7. After 7 days, growing cells were collected and analyzed by flow cytometry for the expression of CD19 and Thy1.2 or CD44 and CD25 on the $Gr1^-CD11b^-NK1.1^-$ fraction to remove myeloid and NK cells in this analysis. (B) Differentiation to the DP stage with Notch signaling mediated by Dll4, D4d-J1 or Dll1. LK-FL cells were cultured on a monolayer of PA6-derived transfectants expressing Dll4, D4d-J1 or Dll1 for 14 days in the presence of Flt3 ligand (5 ng/mL) and IL7 (1 ng/mL). Live cells were then collected and processed for flow cytometric analysis as in Fig. 2 (A). Numbers in quadrants refer to the percentage of populations in each quadrant. Numbers under the profiles represent the cell numbers of $Gr1^-CD11b^-NK1.1^-$ cells ($\times 10^5$/well, A; $\times 10^6$/well, B).

Lfng in modifying Notch signaling. Lfng enhances Notch signaling by Dll1 and suppresses Notch signaling by Jagged1 [22]. Furthermore, Lfng is critical for the development of LK-FL cells into T-lineage cells in the thymus [15–18] where Dll4-mediated Notch signaling is provided [11]. As transcripts of the *Lfng* gene were abundant in LK-FL cells but barely detected in NIH-3T3 (Fig. 4A), we examined the effect of the enforced expression of Lfng on Notch signaling in N1/3T3 cells (Lfng-N1/3T3). The result indicated that the enforced expression of Lfng increased the Notch signaling induced by Dll4 but suppressed that induced by Jagged1 or Jagged2 (Fig. 4B). While Jagged2 continued to induce substantial signaling even in the presence of Lfng, Jagged1-induced signaling became significantly lower than that induced by Jagged2 in Lfng-N1/3T3 (Fig. 4B). Similar results were obtained with the chimeric molecules of Dll4 and Jagged1 (Supporting Information Fig. S1). To estimate the magnitude of actual Notch signaling in HPC, the Hes1 transcript in LK-FL cells was examined by quantitative RT-PCR (Fig. 4C) after culture with stromal cells expressing NotchL. The result indicated that Jagged2 induced substantial Hes1 transcripts while Jagged1 showed a minimal effect in LK-FL cells. This was consistent with the result obtained with Lfng-N1/3T3, and with the ability of each NotchL to support T lymphopoiesis *in vitro*.

The DSL domain has no role in the induction of T cells by Notch ligands

To assess why Jagged1 failed to support T lymphopoiesis, we further examined the function of Jagged1-based chimeras containing Dll4- or Jagged2-derived segments. In comparison with Dll4, the Delta and Jagged families differ considerably in the overall size of their extracellular regions. Jagged molecules possess eight additional (16 in total) EGF-like repeats compared with Dll molecules, as well as a unique cysteine-rich domain of unknown function (Fig. 1A). We designed a Jagged1-based variant with eight EGF repeats (identical to the Dll family) but without the cysteine-rich domain, termed J1 short (Fig. 5A). This variant was detected on the surface by staining with the HRJ1-15 mAb (Fig. 5B) but not anti-Jagged1 mAb (data not shown). J1 short exhibited no difference from Jagged1 in the induction of Notch signaling, even in cells expressing abundant Lfng, and failed to promote T lymphopoiesis (Fig. 5C and D). Furthermore, a shortened Jagged1 variant with a Dll4-derived DSL domain was expressed on the surface at similar levels (Fig. 5B), but did not induce substantial Notch signaling in either N1/3T3 or Lfng-N1/3T3 (Fig. 5C) and did not support T lymphopoiesis (Fig. 5D). These results suggested that half of the extracellular region of Jagged1 is enough to carry out Jagged1 functions, and that the overall size, the cysteine-rich domain of Jagged1 and the DSL domain of Dll4 do not contribute to the functional difference between Dll4 and Jagged1 for T-cell induction.

An additional Jagged1 variant including the Jagged2-derived DSL domain (J2DSL-J1 short) was examined since, like Dll4, the extracellular region of Jagged2 was shown to determine its biological function in the chimera (J2d-J1) (Fig. 5). This variant, expressed at a similar level on the surface, was able to induce greater signaling than Jagged1 in N1/3T3. However, like Jagged1, signaling through J2DSL-J1 short decreased to minimal levels in Lfng-N1/3T3 (Fig. 5C), and J2DSL-J1 short did not

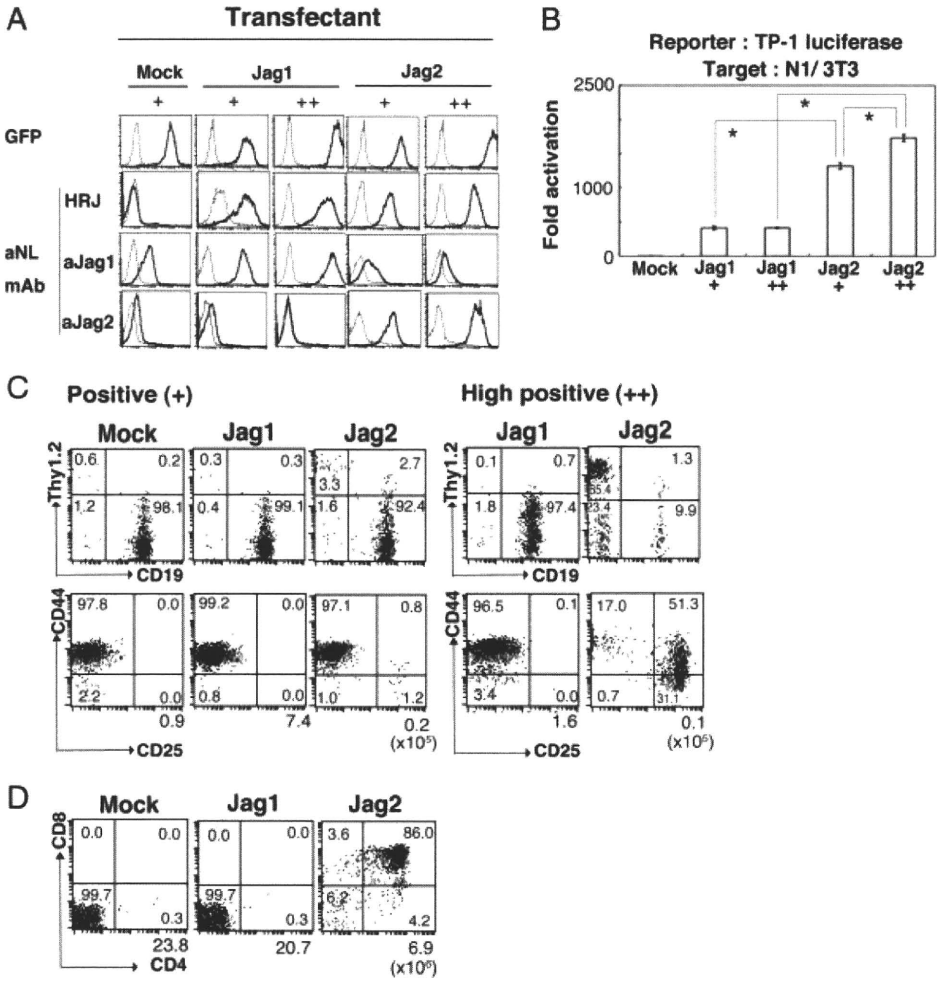

Figure 3. Jagged2 supports T lymphopoiesis *in vitro*. (A) Surface expression of transfected Jagged1 and Jagged2 was examined by flow cytometry with specific antibodies for the ligand (anti-Jagged1, aJ1; anti-Jagged2, aJ2 in lower panels, aNL mAb) or mAb recognizing both Jagged1 and Jagged2 (HRJ1-15, HRJ in lower panels). Thin lines represent the profile with control hamster IgG. For the expression of GFP, thin lines show the control profile of non-transfected 3T3 cells (upper panels, GFP). Several transfectants with different intensities of GFP (positive, +; high positive, ++) were obtained by cell sorting. (B) Jagged2 transduced higher Notch signaling than Jagged1. Notch signaling in N1/3T3 from Jagged1 or Jagged2 was measured as in Fig. 1C. *$p<0.001$, unpaired Student's t test ($n = 3$). Data are representative of five independent experiments. (C) Induction of T-lineage cells by Jagged2 transfectants. The experiment was performed as described in Fig. 2A. (D) Differentiation to the DP stage on PA6-derived transfectants expressing high levels of Jagged1 or Jagged2 as described in Fig. 2B.

support T lymphopoiesis (Fig. 5D). These findings suggested that the DSL domain of Jagged2 has a high potential to induce signaling, but other extracellular regions seem necessary for the Jagged2-like activity with fringe-modified Notch.

Discussion

We show here, using chimeric molecules between Dll4 and Jagged1, that Dll4 but not Jagged1 induces T-cell development *in vitro* and that the extracellular regions of NotchL play a key role in directing T-cell specification. In contrast, intracellular regions are essential but functionally exchangeable between Dll4 and Jagged1. Moreover, both Delta and Jagged family members support T lymphopoiesis, which is dependent on the magnitude of the Notch signaling induced. Thus, not only Dll1 and Dll4, but also Jagged2, promote T-cell development. The functional difference between Jagged1 and Jagged2 could be explained by the magnitude of net signaling through the fringe-modified Notch receptor.

The intracellular region of NotchL was required for signal transduction *via* the Notch receptor but not for their expression. This is consistent with past reports showing that mutants lacking the intracellular region could be expressed on the cell surface but still failed to induce Notch signaling (Fig. 1C) [21, 23]. Moreover, other previous reports showed that the intracellular regions of NotchL interact with E3 ubiquitin ligases, such as Mind-bomb and Neuralized in zebrafish and Drosophila, respectively, which are conserved from Drosophila to humans and promote the endocytosis of Dll1 and Jagged1 [24–27]. In particular, Mib1 (the mammalian ortholog of Mind-bomb) is indispensable for the transduction of Notch signaling *via* NotchL, and Mib1-deficient

