Journal of Immunological Methods 467 (2019) 29–36

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

An improved clonogenic culture method for thymic epithelial cells

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ARTICLE INFO

Keywords:
Thymus
Thymic epithelial stem cells
Colony assay
EGF
LIF

ABSTRACT

A clonogenic assay system for thymic epithelial cells (TECs) is of crucial importance for identifying thymic epithelial stem and/or progenitor cells, evaluating their activities, and understanding the mechanisms of thymic involution. However, current systems are not sufficiently sensitive at detecting and quantifying TEC colonies from the adult thymus. Here, we optimized the culture condition to detect visible colonies from adult TECs by modifying our previous culture methods. Epidermal growth factor and leukemia inhibitory factor significantly enhanced the colony-forming efficiency of total TECs from embryo as well as adult mice when added 3 days after plating. Importantly, characteristics of the TEC colonies formed by the improved condition were almost equivalent to those by the original culture condition with respect to self-renewal and the expression of cell surface markers and intracellular keratins. Furthermore, the colonies derived from total TECs showed immature phenotypes and generated both mature cortical TECs and medullary TECs upon implantation in vivo. These data indicate a more sensitive clonogenic assay system for TECs was established and suggest the improved culture condition supports the colony formation of stem/progenitor cells for cTECs, mTECs and/or bipotent TECs.

1. Introduction

The thymus is the central lymphoid organ essential for T cell development and consists of two distinct anatomical regions, the medulla and cortex. Thymic epithelial cells (TECs) in both regions, medullary TECs (mTECs) and cortical TECs (cTECs), respectively, are major cellular components of a complex thymic microenvironment that controls T-cell proliferation, differentiation, selection, and maturation (Abramson and Anderson, 2017; Takahama et al., 2017).

The existence of TEC stem/progenitors was first demonstrated by the presence of mTEC islets derived from a single cell using major histocompatibility complex (MHC) chimeric mice (Rodewald et al., 2001). Thereafter, the implantation of defined embryonic TEC fractions (MTS24$^+$) into reaggregated thymus under the kidney capsules suggested the existence of common progenitors for cTECs and mTECs at the population level (Bennett et al., 2002; Gill et al., 2002). Bipotent progenitor activity was demonstrated at the single cell level, as the microinjection of a single TEC from embryonic day (Ed12) thymic anlage can generate both mTECs and cTECs (Rossi et al., 2006). Furthermore, a genetic cell-tracing assay in vivo showed single TECs expressing FoxN1 have common progenitor activity in the postnatal thymus (Bleul et al., 2006). Additionally, mTEC progenitors were also identified as claudin (Cld)-3, 4-expressing TECs from Ed13 thymic anlage (Hamazaki et al., 2007).

Colony-forming assays are commonly performed to show the self-renewal activity of tissue stem cells in vitro by propagating the colonies. A two-dimensional (2D) culture system for epidermal stem cells is a successful example, as the cells can be maintained and propagated more than a hundred times (Rheinwald and Green, 1975). On the other hand, neuronal stem cells have been identified using a 3D spheroid culture method (Reynolds and Weiss, 1996). Recently, colony/spheroid-forming assays for TECs have been developed, and TEC stem/progenitor cells were identified in the adult thymus (Hamazaki et al., 2016; Sekai et al., 2014; Ucar et al., 2014; Wong et al., 2014). Common stem/progenitors for mTECs and cTECs were identified utilizing the colony-forming assay in 3D culture with Matrigel and mouse embryonic fibroblasts (Wong et al., 2014). There was also a report that thymosphere cultures are also capable of detecting common thymic epithelial stem cells (TESCs) (Ucar et al., 2014), although whether the spheroids contain mainly TECs requires further investigation (Sheridan et al., 2017).

Abbreviations: cTECs, cortical thymic epithelial cells; Ed, embryonic day; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; IT, intrathymic; LIF, leukemia inhibitory factor; MHC, major histocompatibility complex; mTECs, medullary thymic epithelial cells; mTESCs, medullary thymic epithelial stem cells; TECs, thymic epithelial cells; TESC, thymic epithelial stem cells

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https://doi.org/10.1016/j.jim.2019.02.003
Received 11 September 2018; Received in revised form 28 December 2018; Accepted 5 February 2019
Available online 06 February 2019
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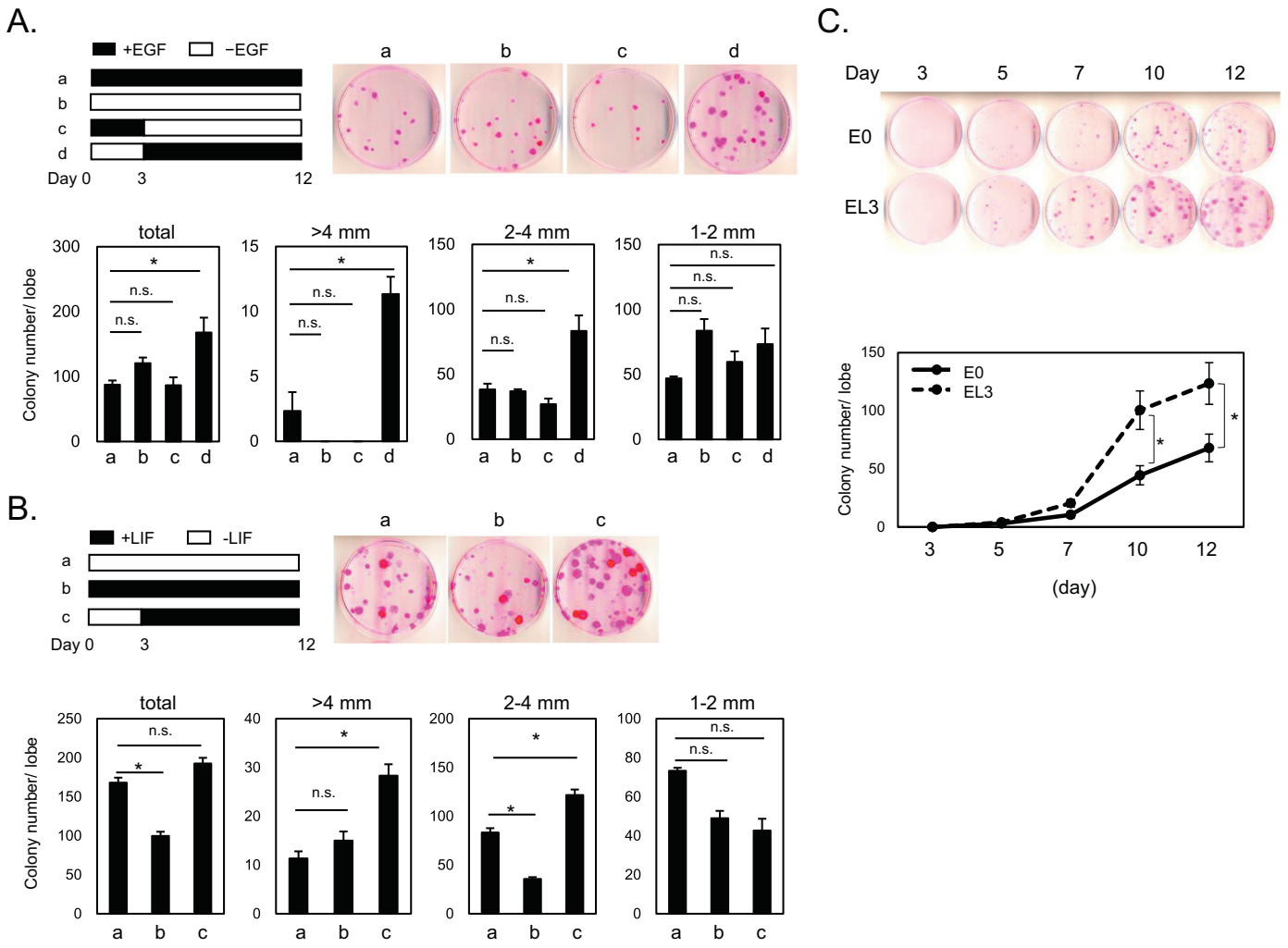


Fig. 1. Effects of EGF and LIF on colony-forming activity.

A. Supplementation conditions of EGF (upper left): a, supplementation of 10 ng mL^{-1} EGF throughout the culture; b, no supplementation of EGF throughout the culture; c, removal of EGF 3 days after plating; d, supplementation of EGF 3 days after plating. Rhodamine-B staining of colonies (upper right) and colony numbers per lobe (lower) in the corresponding 4 conditions (a-d). Statistical analysis was performed using one-way ANOVA, followed by the Holm-Sidak method for multiple comparisons; * $p < .05$, ** $p < .01$, *** $p < .001$, n.s., not significant.

B. Supplementation conditions of LIF (upper left), Rhodamine-B staining of colonies (upper right) and colony numbers per lobe (lower) cultured in EGF condition "d" in (A): a, supplementation with 10^3 U mL^{-1} LIF throughout the culture; b, no supplementation with LIF throughout the culture; c, supplementation with LIF 3 days after plating.

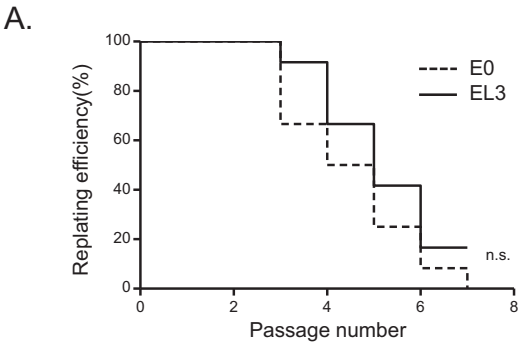
The shown Rhodamine-B staining is representative of at least four independent experiments (A and B). Column data are shown as the mean ± SEM of $n = 3$ and are representative of four independent experiments (A and B). Statistical analysis was performed using one-way ANOVA, followed by the Holm-Sidak method for multiple comparisons; * $p < .05$, ** $p < .01$, *** $p < .001$, n.s., not significant.

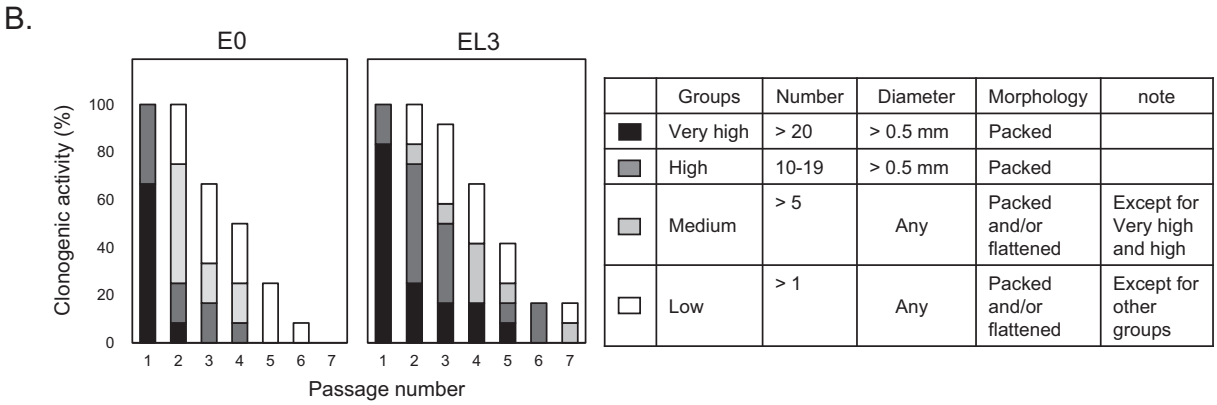
C. Rhodamine-B staining of colonies (upper) and colony numbers per lobe (lower) at the indicated days in E0 and EL3 cultures are shown. Data are representative of three independent experiments. Colony numbers per lobe are shown as the mean ± SEM. Data are pooled from three independent experiments ($n = 4$). Two-tailed *t*-test was performed; * $p < .05$, ** $p < .01$, *** $p < .001$. Ed14 $CD45^-$ thymic cells were used in the assays.

We identified medullary TESCs (mTESCs) by employing a colony-forming assay in 2D culture using a murine embryonic fibroblast cell line, STO, as feeder cells (Hamazaki, 2015; Hamazaki et al., 2016; Sekai et al., 2014).

The thymus begins to involute after puberty, as characterized by a progressive reduction in size and function (Lynch et al., 2009), and the TEC colony-forming activity decreases before visible involution occurs in all assay systems (Kim et al., 2015; Meireles et al., 2017; Sekai et al., 2014). In our assay, the expanded colonies are almost undetectable in young adult mice (Sekai et al., 2014), which makes it difficult to quantitatively analyze thymic epithelial stem cell (TESC) activity during thymic involution. Considering that mTECs are continuously produced from implanted mTESCs in mice in vivo throughout the lifetime of the animal, and colony formation of rat adult TECs is efficiently supported in the same culture system (Sekai et al., 2014), it is possible that the current culture conditions are not fully optimized for mouse TECs.

Therefore, we sought to develop an improved culture method to raise the colony forming efficiency of mouse TECs. We found that the addition of epidermal growth factor (EGF) and leukemia inhibitory factor (LIF) after 3 days of culture significantly increased the absolute number of colonies with indistinguishable cellular phenotypes and self-renewing capacity from the culture condition of our previous method. Importantly, the implantation of colony cells from total TECs produced both mTECs and cTECs, suggesting that this culture condition supports the colony formation of mTECs and cTECs and/or common TEC progenitor/stem cells.





	Groups	Number	Diameter	Morphology	note
■	Very high	> 20	> 0.5 mm	Packed	
■	High	10-19	> 0.5 mm	Packed	
■	Medium	> 5	Any	Packed and/or flattened	Except for Very high and high
□	Low	> 1	Any	Packed and/or flattened	Except for other groups

Fig. 2. Self-renewal activities of colony cells in vitro.
A. Replating efficiencies in propagated cultures from colonies developed in E0 (dashed line) and EL3 (continuous line) cultures. Log-rank test was performed; n.s., not significant.
B. Subtypes of the clonogenic activities shown in (A) (left). Criteria for subtyping the TEC colonies (right).
Data are from a single experiment representative of three independent experiments (n = 12, each group per experiment). Ed14 CD45$^-$ thymic cells were used in the assays.

2. Materials and methods

2.1. Animals

C57BL/6 (B6) and enhanced green fluorescent protein-transgenic (EGFP Tg) B6 mice (Okabe et al., 1997) were obtained from SLC Japan. All animals were maintained under specific pathogen-free conditions at the Institute of Laboratory Animals, Kyoto University, and animal experiments were conducted in accordance with the guidelines for animal experiments of Kyoto University.

2.2. Cell culture

STO cells (obtained from RIKEN BRC, RCB0536) were maintained in DMEM supplemented with 10% feral calf serum (FCS) (Hyclone, USA) and penicillin/streptomycin (Gibco, UK) on tissue culture dishes covered with 0.1% gelatin (Sigma, USA). STO cells were treated with 10 μg mL^{-1} mitomycin-C (Kyowa Hakko Kirin, Japan) and plated at a density of 2.5 × 10^4 cells cm^{-2} in a 60 mm dish before being used as feeder cells.

2.3. Thymic stromal cell isolation

Thymic stromal cell isolation was performed as previously described (Seach et al., 2012; Sekai et al., 2014). Briefly, thymic lobes of Ed14, 1-, 2- and 4-wk-old mice were cleaned of associated fats and minced into small pieces. Except for Ed14 thymi, thymic fragments were gently agitated to release thymocytes. After settling the fragments, the thymocyte-rich fraction was discarded, and the remaining fragments were digested using 0.5 U mL^{-1} Liberase TM (Roche, Germany) and 0.2 mg mL^{-1} DNase I (Roche, Germany) in RPMI media at 37 °C for 10 min with gentle agitation every 5 min into single-cell suspension. After settling the fragments, the supernatants were transferred to a new tube, and the remaining fragments, which were enriched with TECs, were further digested with the enzymes at 37 °C for 10 min as described above. The supernatant and TEC rich fraction were pooled and passed through a 70 μm filter. The cell suspension was centrifuged and re-suspended in PBS supplemented with 5 mM EDTA and 1% FCS (hereafter FACS buffer). Cells were blocked with anti-FcγRIIb for 20 min at 4 °C and incubated with anti-mouse CD45 MicroBeads (Miltenyi Biotec, Germany) for 20 min at 4 °C according to the manufacturer's instruction. CD45$^-$ cells were enriched using AutoMACS (Miltenyi Biotec, Germany). The number of CD45$^-$ cells was 2–5 × 10^4 in Ed14 and 0.3–1 × 10^6 cells in other ages, and the purity of CD45$^-$ cells was > 90%.

2.4. TEC clonogenic assay

Single-cell suspensions of the total CD45$^-$ thymic cells indicated above were cultured in 60 mm dishes on a monolayer of STO feeder cells pretreated with 10 μg mL^{-1} mitomycin-C for 2 h at 37 °C in serum-free medium, 3:1 mixture of DMEM and Ham's F-12, and supplemented with 10% KnockOut Serum Replacement (KSR) (Invitrogen, USA), 0.4 μg mL^{-1} hydrocortisone, 10^{-10} M choleratoxin (Wako, Japan), 5 μg mL^{-1} insulin (Sigma, USA), 2 × 10^{-9} M 3,3,5-triido-L-thyronine (Sigma, USA), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Using our previous method (E0 culture), cells were cultured in serum-free medium with 10 ng mL^{-1} recombinant mouse epidermal growth factor (EGF) (Invitrogen, USA) throughout the culture (Sekai et al., 2014). For the new method (EL3 culture), the serum-free medium was

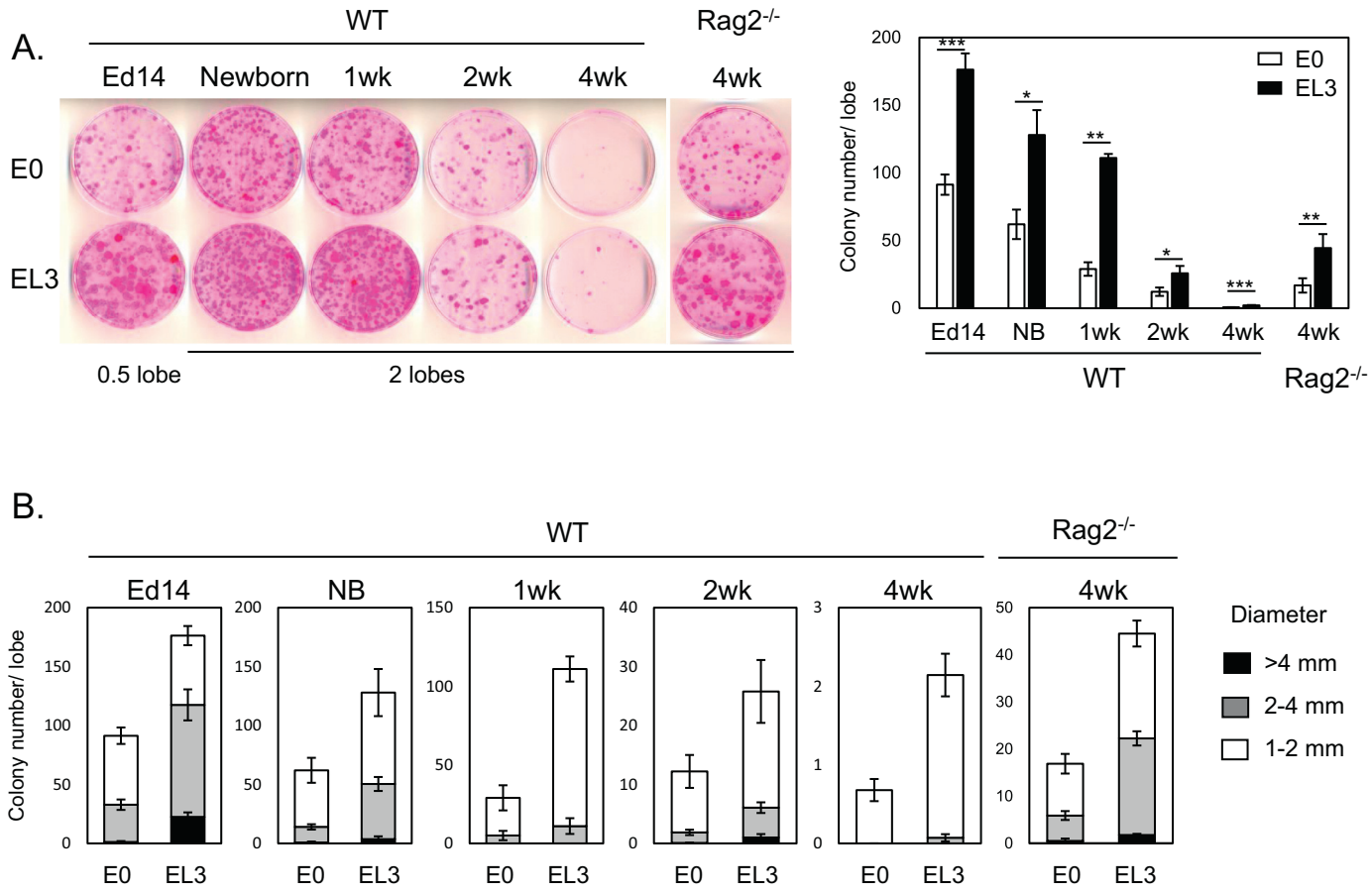


Fig. 3. Clonogenic activities from fetal to adult thymus.

A. Rhodamine-B staining of TEC colonies developed from the indicated aged thymi in E0 and EL3 cultures (left). Images are representative of two independent experiments (at least $n = 5$ each group). Total colony number per lobe in E0 and EL3 cultures (right). Two-tailed t-test was performed; * $p < .05$, ** $p < .01$, *** $p < .001$.

B. Colony numbers per lobe of the indicated size at each age are shown. Data are at least $n = 5$ at each age and are pooled from two independent experiments.

replaced with serum-free medium plus 10 ng mL^{-1} EGF and 10^3 U mL^{-1} LIF (nacalai tesque, Japan) after 3 days of culture. In both methods, media were changed every 3 days.

To expand the colonies, individual colonies cultured for 10 days (maximum size during the culture, 2–4 mm) were treated with Trypsin-EDTA (0.25% Trypsin plus 1 mM EDTA, Invitrogen, USA) for 5–10 min at 37 °C in cloning cylinders, and the reaction was stopped with Defined Trypsin Inhibitor (Invitrogen, USA). Collected colony cells were passaged onto a fresh feeder layer as described previously (Sekai et al., 2014). After the secondary passage, all colonies derived from a single colony in the first plating were passaged (Supplementary Fig. 1). The clonogenic activity in each passage was assessed under an inverted microscope (Olympus, Japan). To visualize colonies, colonies on day 12 of the culture were fixed with 3.7% formalin for 15 min at room temperature (R.T.) and stained with 1% Rhodamine-B for 1 h at R.T. (nacalai tesque, Japan).

2.5. Antibodies

The following fluorescent and biotinylated antibodies were used for flow cytometry: CD45 (clone 30-F11, eBioscience, USA), CD80 (clone 16-10A1, Biolegend), CD86 (clone GL-1, eBioscience), EpCAM (clone G8.8, Biolegend), Ly51 (clone 6C3, eBioscience), MHC II (clone M5/114.15.2, Biolegend), Ter119 (clone Ter119, Biolegend), UEA-1 (Vector laboratories, USA), K5 (Covance, USA), K8 (clone Troma-1, DSHB, University of Iowa, USA), APC-streptavidine (eBioscience), anti-rat IgG FITC (Southern Biotechnology Associates, USA) and anti-rabbit IgG PE (eBioscience).

2.6. Flow cytometry

Thymic cells in single cell suspensions in FACS buffer were blocked with anti-FcγRIIb antibody for 20 min at 4 °C and incubated with antibodies. To label the non-viable cells, propidium iodide (PI) was added to the cells just before acquisition. Intracellular keratin staining was performed using the BD Cytofix/Cytoperm kit (BD Biosciences, USA) according to the manufacturer's instruction. Ghost dye (TONBO) was used to label non-viable cells. Analysis was performed on a FACSCantoII flow cytometer (BD Biosciences, USA) with FACS DIVA software (BD Biosciences, USA).

2.7. Thymic reaggregation and implantation

Thymic reaggregation was performed as described previously (Anderson et al., 1993; Sekai et al., 2014). Thymic lobes from Ed14 mice were enzymatically treated into single-cell suspension as described in section *2.3 Thymic stromal cell isolation*. Pooled colony cells (1×10^4 cells) prepared from all colonies on day 10 in single-cell suspension were mixed with Ed14 total thymic cells (5×10^5 cells containing approximately 1.5×10^4 EpCAM$^+$ cells) in a 1.5 mL tube followed by centrifugation at 300 *g* for 5 min at 4 °C and complete removal of the medium. The cell pellet was dispersed by adding 1 μL of medium and gently pipetting, then incubated on a filter (Whatman, UK) floated on RPMI-1640 containing 10% FCS, 1 mM sodium pyruvate (Gibco,

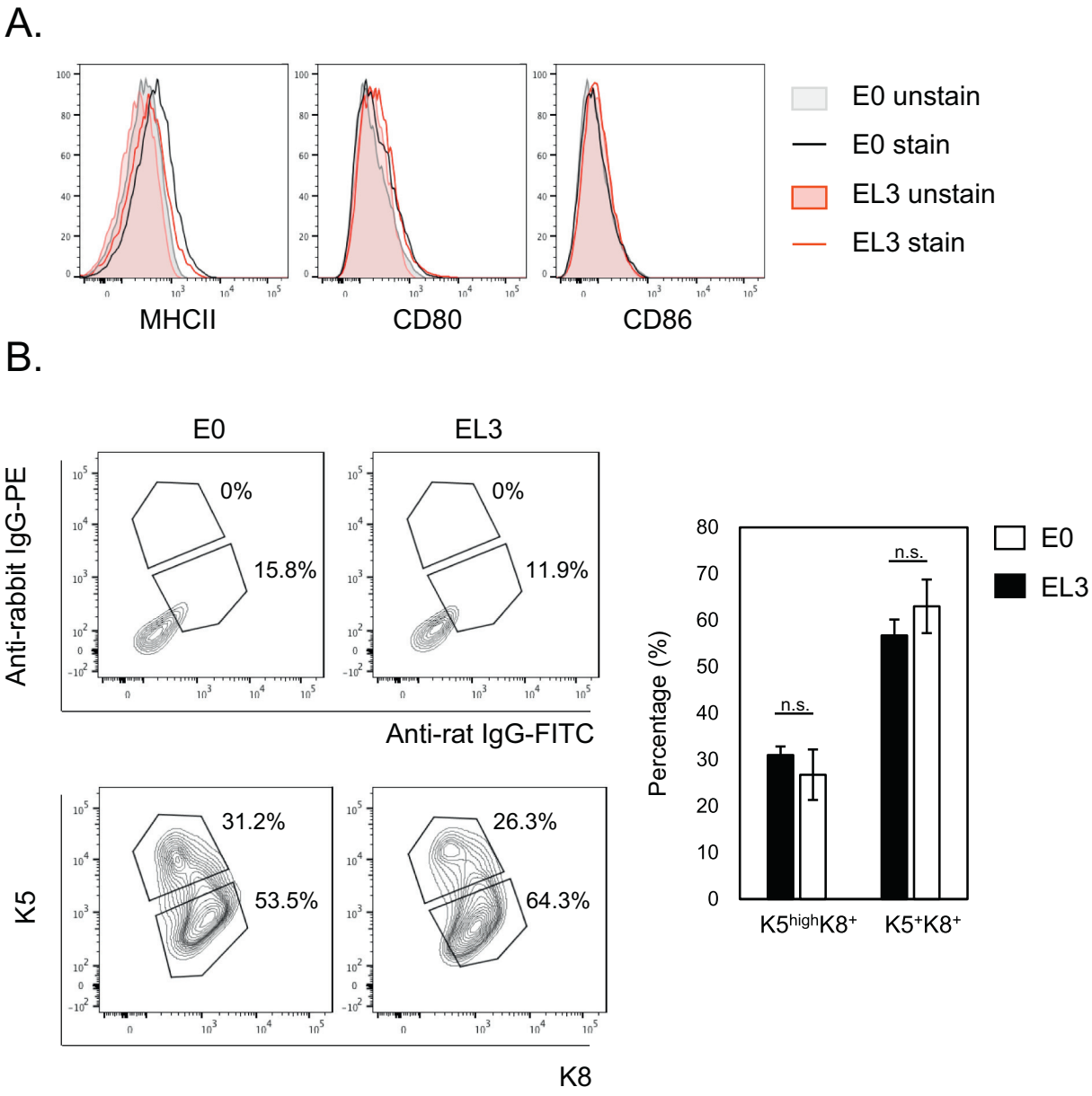


Fig. 4. Cell surface and intracellular molecule expressions in colony cells.
A. Flow cytometry analysis of the expressions of MHC class II (MHC II), CD80 and CD86 in E0 and EL3 colony cells. Colony cells derived from TECs were identified as EpCAM$^+$ cells. Data are representative of three independent experiments.
B. Flow cytometry analysis of the expressions of intracellular keratin 5 and 8 or the 2nd antibody staining in colony cells cultured in either E0 or EL3 (left). Percentage of keratin5^{high} keratin8$^+$ (K5^{high}K8$^+$) and keratin5$^+$ keratin8$^+$ (K5$^+$K8$^+$) in all colony cells identified as EpCAM$^+$ are shown (right). Data are shown as the mean ± SEM of three independent experiments. Two-tailed t-test was performed; n.s., not significant. Ed14 CD45$^-$ thymic cells were used in the assays.

USA), 0.1 mM non-essential amino acids (Gibco, USA), 2-mercaptoethanol (Gibco, USA) and penicillin/streptomycin for 24 h. The resulting cell reaggregates were implanted under the kidney capsules of anesthetized syngeneic mice.

2.8. Intrathymic injection of colony cells

Four-week-old B6 female mice were anesthetized with a vaporized isoflurane system (4 L/min) (DS PHARMA BIOMEDICAL, Japan). The thymus was surgically exposed, and 5×10^5 cells of pooled colony cells derived from Ed14 thymi in 50 µL sterile PBS were injected into each lobe using a 50 µL Hamilton syringe with a 26-gauge bevel-tipped needle (Hamilton, USA).

2.9. Statistics

Statistical analysis was performed using a two-tail *t*-test for the comparison of two means, a one-way ANOVA and multiple t-test where appropriate using SigmaPlot v6.0 software (HULINKS), and a Log-rank test where appropriate using GraphPad Prism v5.0 software (GraphPad Software).

3. Results and discussion

3.1. EGF and LIF enhance colony-forming efficiency of thymic epithelial cells

EGF is commonly supplemented in tissue stem cell cultures,

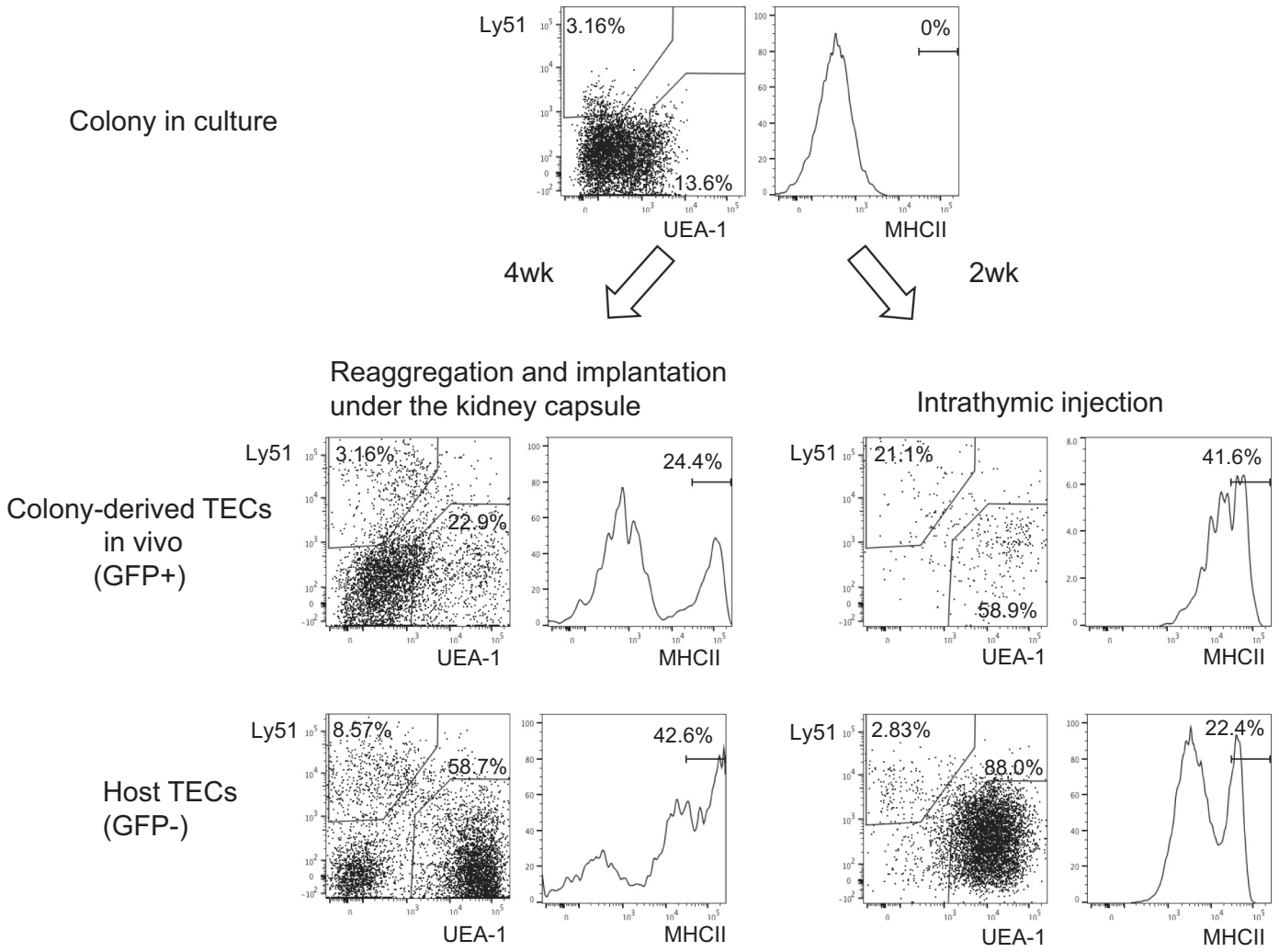


Fig. 5. Differentiation capacity of colony cells in vivo.
Flow cytometry analysis of binding to UEA-1 and expressions of Ly51 and MHC II in EL3 colony cells from CD45- thymic cells of Ed14 EGFP Tg mice on day 10 in culture (Upper), at 4 weeks after reaggregation and implantation (lower left), and at 2 weeks after thymic injection (lower right). Colony cells in culture were identified as $EpCAM^+$ cells. The TEC fraction after the implantation or thymic injection was defined as $CD45^-$ $Ter119^-$ $EpCAM^+$ cells. Data are representative of at least eight independent experiments.

including those for TEC stem/progenitor cells, to support cell growth (Blanpain et al., 2004; Pinto et al., 2013; Rios et al., 2014; Sato et al., 2009; Sekai et al., 2014; Shackleton et al., 2006; Ucar et al., 2014; Wong et al., 2014). Accordingly, we added EGF throughout E0 culture (Sekai et al., 2014). However, EGF reduces the colony-forming efficiency of human epidermal keratinocyte cell lines when added on the first plating (Rheinwald and Green, 1977), even though it is essential for TEC survival in vivo (Jain et al., 2017). To re-assess the effect of EGF supplementation on our system, colony-forming activities were compared in the presence or absence of EGF in various culture conditions using Ed14 thymi. Colony numbers per lobe were not different when EGF was depleted from the whole culture or after the first 3 days (Fig. 1A). However, depleting EGF in the first 3 days of culture enhanced the numbers of total, large (> 4 mm diameter)-, and medium (2–4 mm diameter)-sized colonies (Fig. 1A).

LIF is commonly used in culture to maintain undifferentiated embryonic stem (ES) and induced pluripotent stem (iPS) cells (Niwa et al., 2009; Takahashi et al., 2007). We examined the effect of LIF on the culture, in which EGF was depleted in the first 3 days. Unexpectedly, the addition of LIF throughout the culture significantly decreased the colony number (Fig. 1B). However, the depletion of LIF in the first 3 days of culture (EL3 culture) significantly enhanced the colony number of large- and medium-sized colonies, although the total colony number was unchanged (Fig. 1B). Importantly, almost all non-feeder cells expressed EpCAM, confirming that the colonies contained only TECs (Supplementary Fig. 1). Kinetic analysis indicated that the increased number of colonies in EL3 culture compared with E0 culture became significant around 10 days after plating (Fig. 1C). These results indicated that EGF and LIF supplementation 3 days after plating significantly enhances TEC colony formation, suggesting that EGF and LIF may suppress the settling and/or expansion of TECs on feeder cells at the beginning of the culture, but simultaneously support the survival and proliferation of TECs after 3 days in culture.

3.2. TEC colonies developed with LIF show self-renewal ability

Self-renewal is an essential criterion of stem cells and is estimated in vitro as the propagation ability of colonies derived from a single cell (Gambardella and Barrandon, 2003; Martello and Smith, 2014). We passaged single cell-derived TEC colonies using either E0 or EL3 culture on fresh feeder cells (Supplementary Fig. 2) and compared their "replating efficiency", which is indicated by the proportion of passaged colony numbers per initial input colony number (Sekai et al., 2014). All TEC colonies derived in E0 culture were propagated at the first and

second passages (i.e. 100%), but the efficiency was only 66.7% at the third passage (8 of 12 colonies), and 8.3% at the sixth passage (1 of 12 colonies) (Fig. 2A). All TEC colonies derived in EL3 culture were also propagated at the first and second passages, but 91.7% at the third passage (11 of 12 colonies) and 16.7% at the sixth passage (2 of 12 colonies) (Fig. 2A). TEC colonies derived from E0 and EL3 cultures showed similar clonogenic efficiency at each time point and were passaged up to 7 times, indicating comparable self-renewal ability (Fig. 2A). To assess the "clonogenic activity" at each passage point, colonies were grouped in terms of colony number, morphology, and size as follows: Very high, > 20 packed colonies > 0.5 mm in diameter; High, 10–19 packed colonies > 0.5 mm in diameter; Medium, > 5 packed and/or flattened colonies of any size, but unqualified for Very high or High; and Low, at least 1 packed and/or flattened colony of any size, but unqualified for the other three groups. Although the replating efficiencies were comparable (Fig. 2A), the clonogenic activity at each time point was higher in EL3 culture compared with E0 culture (Fig. 2B). We found most colonies in either culture were categorized as "Very high" or "High" at the first passage (Fig. 2B), but the clonogenic activities decreased with passaging (Fig. 2B). However, EL3 culture showed higher clonogenic activity at all time points, and High colonies were observed even at the fifth and sixth passage, but were absent in E0 culture (Fig. 2B). These results indicate that the newly established EL3 culture condition supports the self-renewing activity of TECs in vitro at a level comparable with that of E0 culture, but that it also enhances the expansion of individual colonies.

3.3. TEC clonogenic activity decreases with age

The number of TECs and proportion of $Ki67^+$ proliferating TECs decrease during age-dependent thymic involution, and these changes precede the actual decrease of thymic mass, which begins at around 4 weeks in mice (Gray et al., 2006). We previously demonstrated that TEC clonogenic activity was dramatically decreased soon after birth, and visible colonies were hardly detected in the young adult stage of mice even though the thymus is still functional (Sekai et al., 2014). Similar phenomena were observed in other studies using different clonogenic assay systems for mouse TECs (Kim et al., 2015; Meireles et al., 2017). Under EL3 culture, the total colony number was significantly increased compared to E0 culture at any stage examined (Ed14 to 4-wk-old), and an age-dependent decrease in colony number was observed using EL3 culture similarly to EL0 culture (Fig. 3A and Supplementary Fig. 3). Additionally, large- and medium-sized colonies were almost undetectable in 4-wk-old mice in both conditions (Fig. 3B). Noteworthy, however, small (1–2 mm in diameter) colonies were visible in 4-wk-old thymi using EL3 culture (Fig. 3B). Importantly, $Rag2^{-/-}$ thymi of 4-wk-old mice also showed an increased number of total colonies, especially large- and medium-sized colonies, in EL3 culture compared to E0 culture, and much higher clonogenic activity than WT thymi at the same age, as we reported previously (Sekai et al., 2014) (Fig. 3 and Supplementary Fig. 3). These results indicated that EL3 culture was sufficiently sensitive for detecting the age-dependent decrease in the clonogenic activity of TECs and TEC colonies from adult thymi.

3.4. Colony cells in both E0 and EL3 cultures show similar phenotypes

Colony cells in TEC colonies generated in E0 culture show immature phenotypes (MHC II^{low} and $CD80/86^{low}$) and express maturation markers such as MHC II upon in vivo implantation (Sekai et al., 2014). We examined the phenotypes of colony cells developed under EL3 culture. The colony cells in E0 and EL3 cultures showed similar phenotypes with no or low expression of the maturation markers MHC II, CD80 and CD86 (Fig. 4A). The TEC phenotype is also characterized by keratin 5 (K5) and keratin 8 (K8) expression; immature TECs show K5 and K8 double positive (DP) phenotype, and K5 and K8 are expressed mainly in mTECs and cTECs, respectively (Bennett et al., 2002; Klug et al., 1998). Flow cytometry analysis demonstrated that most of the colony cells in either E0 or EL3 culture expressed both K5 and K8 (Fig. 4B). In addition, approximately 30% (31.0 ± 3.20% in E0 and 26.8 ± 9.41% in EL3) (mean ± s.d.) and 60% (56.7 ± 5.95% in E0 and 63.0 ± 9.99% in EL3) of them were $K5^{high}K8^+$ and $K5^+K8^+$, respectively (Fig. 4B and Supplementary Fig. 4). These results suggest that colonies formed in both E0 and EL3 cultures show similar phenotypes and consist of two heterogeneous populations in terms of the keratin expression pattern.

3.5. Colony cells differentiate into mature TECs in vivo

Using E0 culture, we demonstrated that TEC colonies developed from the defined TEC fraction (Claudin3,4^{high} SSEA-1^+) of Ed14 thymi showed self-renewing activity and differentiated into mature mTECs in vivo, indicating that E0 culture can support TEC colonies developed from a mTESC (Sekai et al., 2014). However, it remains unknown whether our culture system can support colonies developed from cTEC or common stem/progenitor cells. Thus, we examined the differentiation potential of colony cells derived from Ed14 total TECs using EL3 culture. The phenotypes of colony cells and those after implantation in vivo were evaluated based on UEA-1 binding (mTECs) and the expressions of Ly51 (cTECs) and MHC II (mature mTECs) (Farr and Anderson, 1985; Klug et al., 1998; Surh et al., 1992). The colony cells were negative or low for UEA-1-binding and Ly51 expression, and hardly expressed MHC II in vitro (Fig. 5, upper), suggesting they are not committed to either cTEC or mTEC lineage. To validate the differentiation potential of the colony cells, pooled colony cells in a primary culture developed from the Ed14 thymi of EGFP KI mice were transplanted in vivo by means of a reaggregation-transplantation system or intrathymic (IT) injection. In the reaggregation-transplantation system, a portion (22.3 ± 16.6%) of colony-derived cells ($EGFP^+$) became $Ly51^+$ or UEA-1^+ and MHC II^{high} (Fig. 5, lower left), indicating that they differentiated into cTECs or mTECs. There was no significant difference in the percentage of colony-derived ($EGFP^+$) cells cultured in E0 and EL3 and recovered after the implantation (Supplementary Fig. 5). However, we could not compare the recovery ratio (recovered $EGFP^+$ cell numbers per input cell numbers) because of the low incorporation frequency of colony cells into an organoid in a reaggregation culture. Two weeks after IT injection, almost all colony-derived cells became either $Ly51^+$ cTECs or UEA-1^+ mTECs, and UEA-1, Ly51 double negative immature TECs were almost absent, suggesting that the IT injection system supported TEC differentiation more efficiently than the reaggregation system (Fig. 5, lower right). Indeed, approximately half (47.9 ± 9.26%) of colony-derived TECs were MHC II^{high} (Fig. 5, lower right). These results suggested that EL3 culture can support the proliferation and expansion of colony cells that have the potential for differentiating into mature cTECs and/or mTECs in vivo. In other words, the culture supports cTEC stem/progenitors, mTEC stem/progenitors, and/or biopotent stem/progenitors (Sekai et al., 2014; Ucar et al., 2014; Wong et al., 2014).

4. Conclusion

A sensitive and reproducible culture system for detecting TEC colonies is crucial for the quantitative analysis of TEC stem/progenitor cells. The supplementation of EGF and LIF 3 days after plating greatly enhanced TEC colony-forming efficiency based on the number of visible colonies detected from total TECs of the adult thymus. This improved culture system will enable us to investigate the phenotypic and functional changes of TEC stem/progenitor cells with age and the signaling pathways regulating the activities of these cells.

Declarations of interest

None.

Funding sources

This work was supported by grants from AMED under Grant Number (JP18gm5010001) to Y.H., iPS Cell Research Fund to Y.H., the Japanese Ministry of Education, Culture, Science, Sports, and Technology (24590580, 18H02640 to Y.H. and 15K19125 to M.S.), Grant in Aid for Scientific Research on Innovative Areas “Stem Cell Aging and Disease” (17H05641 to Y.H.) and "Analysis and Sythesis of Multidimensional Immune Organ Network" (15H01154 to Y.H.) from Ministry of Education, Culture, Sports, Science and Technology of Japan, the Takeda Science Foundation to Y.H. and M.S., and the Sumitomo Foundation to M.S.

Acknowledgements

We thank Dr. P. Karagiannis for proofreading.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.02.003.

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