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Supplemental Information

Identification of a Bipotent Epithelial Progenitor Population in the Adult Thymus

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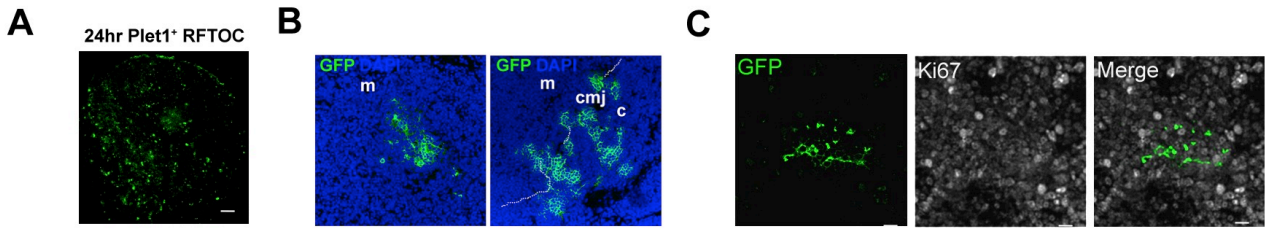
Supplemental Figures and Tables

Figure S1, related to Figure 3: Validation of an assay for functional analysis of postnatal thymic epithelial cells.



(A) Dispersed distribution of GFP^+ cells after 24 hours of reaggregation. (**B,C**) Analysis of grafts recovered after 4 weeks showed clusters of GFP cells ranging from 2-200 cells. **(B)** Cells were found in the medulla (m), cortex (c), and cortical medullary junction (cmj). **(C**) Some GFP^+ cells within the grafts were actively proliferating, as shown by Ki-67 staining. **(A-C)** Images are representative of seven separate sorting and grafting experiments. Scale bars, **A** 100μm; **C,** 25μm. n≥7. Data shown are for total GFP^+PLET1^+TEC; similar data were obtained from GFP^+PLET1^-TEC.

Table S1: Summary of distribution of GFP^+ cells in graft seeded with 90 input Ly-$51^+UEA1^-PLET1^+$ TEC, related to Figure 4.

Section number	GFP^+ Areas
1-96	-
97-102	C1
103-166	-
167	M1
168-173	C2, M1
174-188	M1
189-240	-

Graft was recovered from the kidney and processed for cryosectioning. 8μm sections were cut and every section was captured. Every section was then screened for the presence of GFP^+ cells. When foci of GFP^+ cells were observed, the sections were stained for mTEC and cTEC markers to determine the identity of the GFP^+ cells. GFP^+ foci were tracked through sections, and the number of sections spanned and the position of foci in the graft relative to other GFP^+ foci was noted. Three GFP^+ areas were detected in this graft and are assumed to originate from a single cell. Nomenclature: C1, C2, cortical GFP^+ areas, M1, medullary GFP^+ area. The section numbers in which each area was detected by manual tracking are noted.

Table S2. Antibodies used for IHC and flow cytometry, related to Figures 1-6 and Tables 1 and 2.

Antibody	Clone(s)	Supplier	Staining
AIRE	M-300, D17	SCBT	IHC
Beta5t	Polyclonal	MBL International	IHC
CD11b FITC	M1/70	E-Bioscience	FC – Lineage
CD11c FITC	HL3	E-Bioscience	FC – Lineage
CD11c FITC	HL3	E-Bioscience	FC – Lineage
CD11c PerCP-Cy5.5	418	E-Bioscience	FC – Lineage
CD205	NLDC-145	AbD Serotec	IHC
CD3ε FITC	145-2C11	E-Bioscience	FC – Lineage
CD4 FITC and PerCP-Cy5.5	RM4-5	BD Biosciences	FC – Lineage
CD31-FITC	390	BioLegend	FC – Lineage
CD31		BD Biosciences	FC – Lineage
CD45 PerCP-Cy5.5	30-F11	E-Biosciences	FC – Lineage
CD8α FITC and PerCP-Cy5.5	53-6.7	E-Bioscience	FC – Lineage
CLDN4	Polyclonal	Gift from M. Furuse and S. Tsukita, Kyoto University	IHC
Cytokeratin 5 (AF 138)	Polyclonal	Covance	IHC
Cytokeratin 14 (AF 64)	Polyclonal	Covance	IHC
Cytokeratin 14	LL002	Gift from E.B. Lane, University of Dundee	IHC
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)		Invitrogen	Viability dye
EpCAM APC-Cy7	G8.8	BioLegend	FC
GFP	Chicken polyclonal	AbCam	IHC, FC
GFP		Molecular Probes	FC
Ki-67	15580, 66185	AbCam	IHC
Ly-51	6C3	BioLegend	IHC
Ly-51 PE	6C3	Biolegend	FC

MHC Class II PE-Cy7	M5/114.15.2	BioLegend	FC
MHC Class II biotin	2G9		FC
Pan-Cytokeratin	Polyclonal	DAKO	IHC
PDGFR-α		BD Biosciences	FC – Lineage
PLET1	1D4	In house	IHC, FC
PLET1	MTS20	Gift from RL Boyd, Monash University	FC
PLET1	MTS24	Gift from RL Boyd, Monash University	IHC
RAC1	23A8	Upstate Biotechnology	IHC
TCRβ PerCP-Cy5.5	H57-597	BioLegend	FC – Lineage
Ter119 FITC and PerCP-Cy5.5	Ter119	E-Bioscience	FC – Lineage
UEA-1 biotin	Lectin	Vector Labs	IHC
Brilliant Violet 650 - Streptavidin		Biolegend	FC
Goat anti-rat Alexa Fluor 647		Life Technologies	FC

Table S3. Sequences of primers for QRT-PCR, related to Figure 2.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Aire	GGTTCCTCCCCTTCCATC	GGCACACTCATCCTCGTTCT
Bp-1	TGGACTCCAAAGCTGATCCT	TCAGCCCATCTGACTGGAAT
Cd80	TCGTCTTTCACAAGTGTCTTCAG	TTGCCAGTAGATTCGGTCTTC
Dll4	AGGTGCCACTTCGGTTACAC	GGGAGAGCAAATGGCTGATA
Foxn1	TGACGGAGCACTTCCCTTAC	GACAGGTTATGGCGAACAGAA
Hmbs	TCCCTGAAGGATGTGCCTAC	ACAAGGGTTTTCCCGTTTG
Hprt	CCTCCTCAGACCGCTTTTT	AACCTGGTTCATCATCGCTAA
Il7	CGCAGACCATGTTCCATGT	TCTTTAATGTGGCACTCAGATGAT
Kitl	TCAACATTAGGTCCCGAGAAA	ACTGCTACTGCTGTCATTCCTAAG
Krt5	CCTTCGAAACACCAAGCAC	GTTCTGGAGGTTGGCACACT
Krt14	ATCGAGGACCTGAAGAGCAA	TCGATCTGCAGGAGGACATT
Ltbr	GCTCCAGGTACCTCCTACTCG	ATGGCCAGCAGTAGCATTG
Tp63	GGAAAACAATGCCCAGACTC	AATCTGCTGGTCCATGCTGT
Pax1	CTCCGCACATTCAGTCAGC	TCTTCCATCTTGGGGGAGTA
Plet1	CATCCGTGAAAATGGAACAA	CATCCGTGAAAATGGAACAA
Sca1	ATGGCCAGCAGTAGCATTG	TTACTTTCCTTGTTTGAGAATCCAC
Ywhaz	AACCTGGTTCATCATCGCTAA	GGGTTTCCTCCAATCACTAGC

Supplemental Experimental Procedures

MEF: Murine embryonic fibroblasts (MEF) were prepared from E13.5 CBAxC57BL/6F1 embryos stripped of all internal organs and trypsinized into a single cell suspension, as previously described (Bennett et al., 2002). Cells were plated in DMEM containing 10% fetal calf serum, 50U/ml penicillin, 50ug/ml streptomycin and Glutamine/Pyruvate. Cells were used after a minimum of 3 days and maximum of 3 passages.

Immunohistochemistry: Immunohistochemistry was performed as described (Gordon et al., 2004). Frozen sections 8μm in thickness were serially cut onto slides coated with poly-L-lysine (Sigma), air-dried and then stored at -800C overnight prior to staining. Sections were fixed briefly in cold acetone, washed with PBS and blocked in serum-free protein block (Dako) or 5 % Goat serum for 45 minutes at room temperature. Sections were then incubated with primary antibody for 1 hour followed by the appropriate secondary antibody for 45 minutes. Sections were mounted with Vectashield (Vector labs). Appropriate isotype and negative controls were included in all experiments. DAPI was used as a nuclear counterstain. For detection of immunofluorescence, slides were examined with a Leica AOBS (Leica Microsystem, GmbH) or SPE confocal microscope. The images presented are either single optical sections or projected focus stacks of serial optical sections.

Antibodies: The antibodies used for immunohistochemistry and flow cytometry were as listed in Table S2. Unconjugated mAbs were detected by using mouse a-rat IgM-PE, goat a-rat IgG-Alexa 488, 568 & 647, goat a-rabbit IgG-Alexa 488, 568 & 647, goat a-mouse IgG-Alexa 488, 568 & 647, streptavidin 488, 568 & 647 (all Molecular Probes) and SAV V450 streptavidin (BD Biosciences) and BV 650 (Biolegend). Isotype-control antibodies were all from BD BioSciences.

Flow Cytometry: For the experiments in Figure 3, adult thymi were gently digested by serial incubations in 0.125% (wt/vol) collagenase (Sigma) followed by 0.125% (wt/vol) CollagenaseD/dispase (Roche), both supplemented with 100 units of DNaseI (Invitrogen) at 370C. Single cell suspensions were serially stained and PLET1+CD45-CD31-Ter119-PDGFRα- cells and PLET1-CD45-CD31-Ter119- PDGFRα- were collected, without prior depletion of CD45+ cells or selection of EpCam+ cells, using a MoFlo (DakoCytomation) or FACS ARIA II (BD). Sorted fractions were >95% pure and cytospin analysis confirmed that all PLET1+ and most PLET1- sorted cells were epithelial. For all other experiments, adult thymi were dissociated in RPMI-1640 HEPES media (Invitrogen) with 1.25mg/ml collagenase D (Roche) and 0.05mg/ml DNase I (Roche) for 3x15 minutes at 37oC. Any remaining fragments of tissue were resuspended in RPMI-1640 HEPES media with 1.25mg/ml collagenase/dispase (Roche) and 0.05mg/ml DNaseI (Roche) for a further 30 minutes. CD45+ cells were depleted prior to sorting using CD45 microbeads (Miltenyi Biotech; at 4-5μl of magnetic microbeads per 107 cells). The CD45+ fraction was removed using LS columns (Miltenyi Biotech) and a QuadroMacs or AutoMACS separator (Miltenyi Biotech). The CD45- fraction was collected by centrifugation and then resuspended in the appropriate antibody cocktails for staining. Any remaining T-cells and other non-TEC stroma were excluded and TEC were selected based on positive EpCAM staining. The TEC fraction was subdivided into mTEC and cTEC using UEA1 and Ly51 respectively, and then further subdivided based on PLET1 expression and level of MHC II expression. 7AAD or DAPI was used to identify dead cells. Isotype controls and purity checks were run with each experiment. Sorts were performed on a MoFlo (DakoCytomation; for data shown in Fig. 3), or a Facs Aria II (all other data), All flow cytometry data were analysed using FlowJo Version 9.7.6 (Tree Star, Inc). QRT-PCR: 50 cells were sorted into 10μl CellsDirect 2x Reaction Mix (Invitrogen) containing 4U SUPERase In RNase inhibitor (Ambion). cDNA was synthesised and pre-amplified in one step by addition of 1μl CellsDirect SuperScript III reverse transcriptase / Platinum Taq mix (Invitrogen) and target-specific primers at a final concentration of 50nM. Thermal cycling was performed as follows: 50oC for 15 minutes; 95oC for 2 minutes; 18 cycles of 95oC for 15 seconds, 60oC for 4 minutes. The relative expression level of the target genes was determined using a LightCycler 480 real-time PCR instrument (Roche) and the Roche Universal Probe Library. qPCR was conducted under the following conditions: 95oC for 5 minutes; 40 cycles of 95oC for 10 seconds, 60oC for 20 seconds. Data are shown after normalization to the geometric mean of three control genes (Hprt, Ywhaz, Hmbs). Technical triplicates were run for all samples and no RT and no template controls were included in all experiments. Data analysis was carried out using LightCycler 1.5 software and the DCt method (Livak and Schmittgen, 2001). Primers used for RT-qPCR are as shown in Table S3.