European Journal of Immunology

Supporting Information

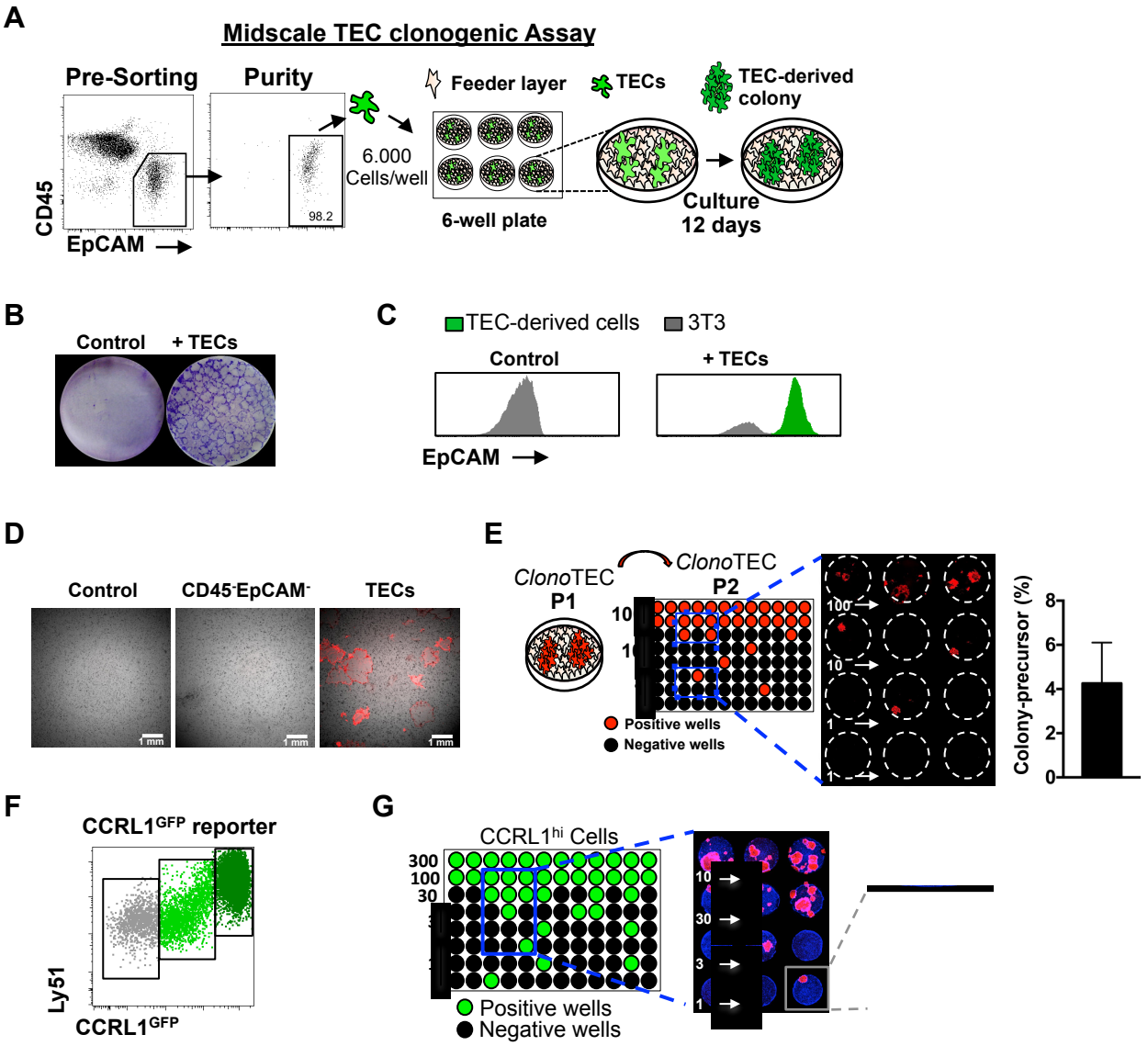
for

DOI 10.1002/eji.201746922

Catarina Meireles, Ana R. Ribeiro, Rute D. Pinto, Catarina Leitão, Pedro M. Rodrigues and Nuno L. Alves

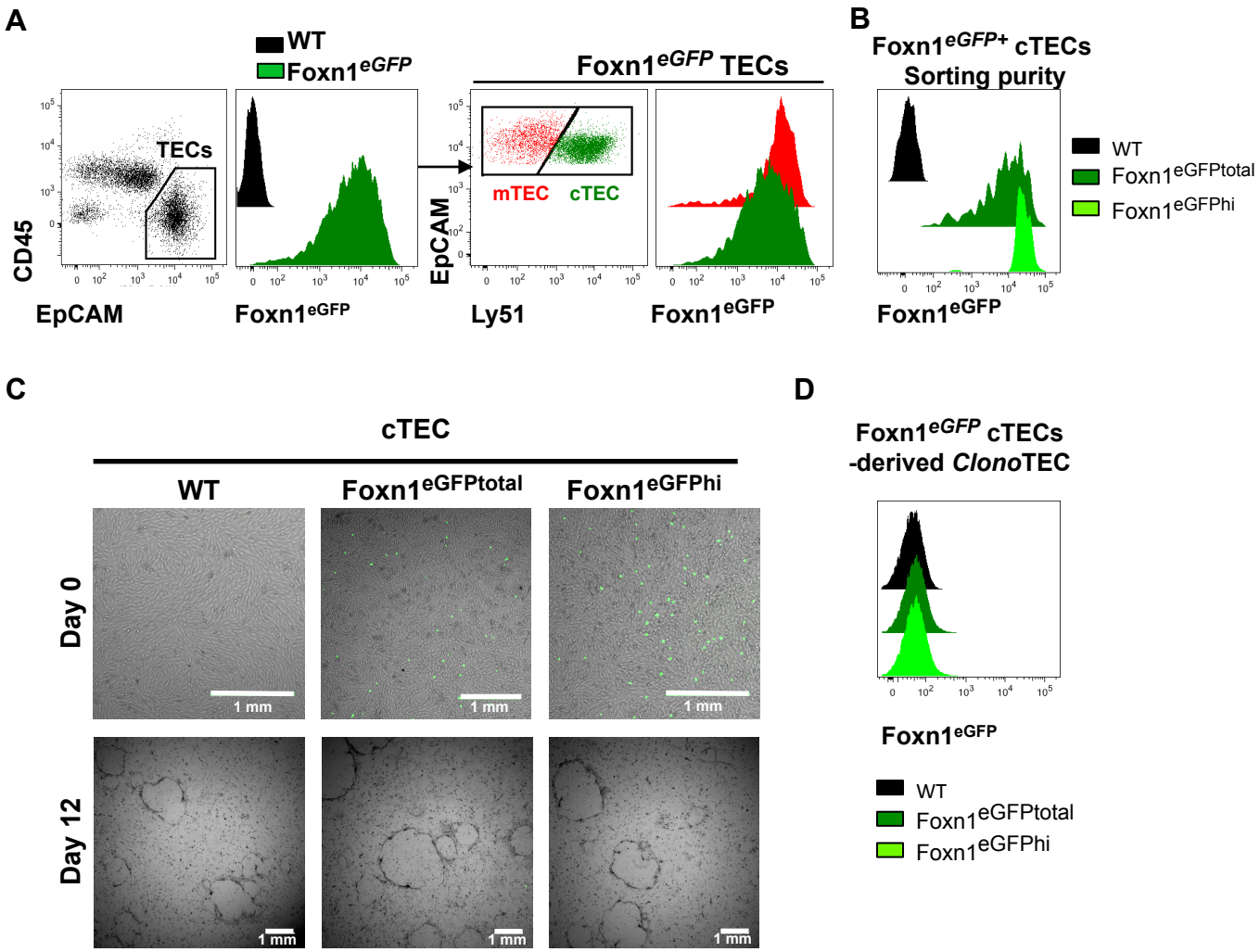
Thymic crosstalk restrains the pool of cortical thymic epithelial cells with progenitor properties

Supporting information Figure 1



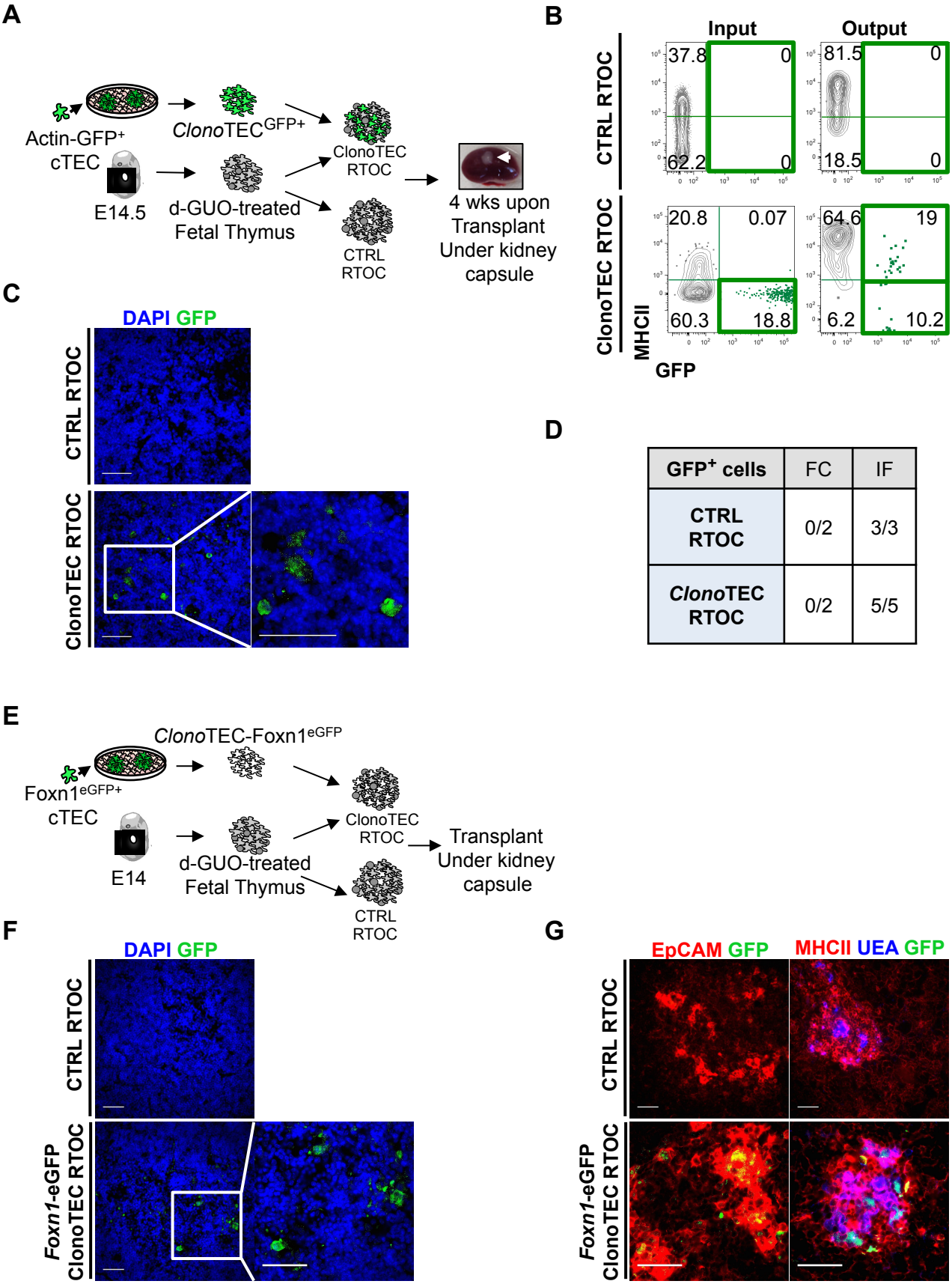
Supporting Information Figure 1. Postnatal cortical (CCRL1^{GFPhi}) thymic epithelial cells (TECs) are enriched in cells with clonogenic capacity. (A) A schematic representation of Midscale Clonogenic Assay. TECs were purified by cell sorting. Flow cytometry analysis of postnatal day 5 (P5) thymus before (Pre-sorting) and after TEC purification by cell sorting (Purity). Six thousand cell-sorted total TECs were seeded onto feeder layer (irradiated 3T3s) in clonogenic assays (as described in Material & Methods) in flat-bottomed 6-well microplates and cultured for 12 days. **(B)** Representative macroscopic images of clonogenic assays in the absence (control) or presence of TECs (+TECs). Hemacolor staining revealed TEC colonies 12 days after culture. **(C)** P5-derived colonies were analyzed by flow cytometry for EpCAM in clonogenic assays set in the absence (control) or presence of TECs (+TECs). **(D)** Six thousand cell-sorted total TECs or non-TEC stromal cells (CD45$^-$EpCAM$^-$) from P5 Actin^{RFP} mice were cultured into midscale clonogenic assay. Control cultures only with feeder cells are shown (Control). The colony formation was determined by bright-field and fluorescence live cell imaging after 12 days in culture. Images represent complete individual wells and are illustrative of three experiments. (**E**) Clonogenic assays were established with cell-sorted TECs from P5 Actin^{RFP} at the indicated density (6.000 cells). 12 days after culture, *Clono*TECs (P1) were analyzed by flow cytometry (EpCAM$^+$RFP$^+$), purified by cell sorting and re-cultured into 96-well microscaled clonogenic assays at the indicated densities (100, 10 and 1 cell per well) (scheme: Red circles mark positive wells for clonogenic activity, black circles mark wells with no activity). Representative images of individual wells are shown. Graph represents the colony-precursor cell frequency (%) of *Clono*TECs from passage 1 (P1), estimated by dividing the number of colonies obtained by the number of seeded TEC (input) x 100. Images represent complete individual wells and are illustrative of three experiments. (**F**) Total TECs (CD45$^-$EpCAM$^+$) from postnatal day 5 (P5) CCRL1^{GFP} reporter mice were analyzed by flow cytometry for CCRL1^{GFP} and Ly51 expression. (**G**) P5 CCRL1^{hi} TECs were sorted at designated densities in a microscaled clonogenic assay (96-well plates) as depicted in the scheme of Microscale Clonogenic Assay. Green circles mark positive wells for clonogenic activity, while black circles mark wells with no activity. Plates were analyzed 12 days after culture for K8 expression to reveal TEC-derived colonies. Images represent complete individual wells and are illustrative of at least five experiments.

Supporting information Figure 2



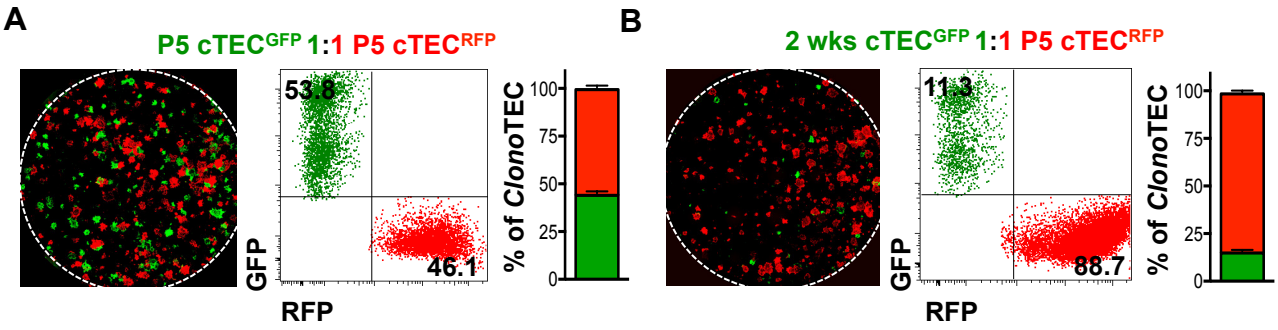
Supporting Information Figure 2. cTECs displaying clonogenic potential lose Foxn1 expression *in vitro*. (A) Left: Total TECs ($CD45^{-}EpCAM^{+}$) from postnatal day 5 (P5) from $Foxn1^{eGFP}$ reporter and WT littermates were analyzed for $Foxn1^{eGFP}$ expression within TECs. Right: cTECs and mTECs from $Foxn1^{eGFP}$ reporter were analyzed for $Foxn1^{eGFP}$ expression. $Foxn1^{eGFP}$ cTECs were cell sorted to establish clonogenic assays. (**B**) Sorting purity of WT, total $Foxn1^{eGFP}$cTEC and $Foxn1^{eGFPhi}$ cTECs is shown. (**C**) Representative microscopy images of clonogenic assays established with cell-sorted WT, total $Foxn1^{eGFP}$ and $Foxn1^{eGFPhi}$ cTECs analyzed at indicated time points. Images are illustrative of at least three experiments. (**D**) WT-, total $Foxn1^{eGFP}$- and $Foxn1^{eGFPhi}$ cTEC-derived *Clono*TECs were analyzed for $Foxn1^{eGFP}$ expression at day 12.

Supporting information Figure 3



Supporting Information Figure 3 (A) A schematic representation of the lineage tracing experiment using Actin^{GFP}-derived *Clono*TECs. Briefly, cTEC^{ActinGFP}-derived *Clono*TECs^{GFP} were purified by cell sorting and aggregated with cells from dGuo-treated E14.5 thymic lobes (*Clono*TEC RTOC) at a ratio 1:8. As control, RTOCs were established with cells from dGuo-treated E14.5 thymic lobes only. (CTRL RTOC). RTOCs were transplanted into the kidney capsule of WT mice, ectopic thymi were recovered 4 weeks post-transplantation and analyzed by flow cytometry **(B)** or fluorescent microscopy **(C)**. **(B)** TECs (CD45^{-}EpCAM^{+}) were analyzed for the expression of MHCII and GFP before reaggregation (input) and after transplantation (output). FACS plots are representative of 3-experiments/ectopic thymus. **(C)** Immunofluorescence analysis of ectopic thymus. Control and *Clono*TECs RTOCs were screened for GFP cells (anti-GFP Ab). Square designates the zoomed area. 50μm scale is shown. Images are representative of 5 ectopic thymus containing *Clono*TEC^{GFP}. **(D)** Table represents the number of RTOCs analyzed by flow cytometry (FC) or fluorescent microscopy/immunofluorescence (IF) in which *Clono*TECs^{GFP}-derived GFP^{+} cells were found. **(E)** A schematic representation of the lineage tracing experiment using Foxn1^{eGFP}-derived *Clono*TECs. Briefly, cTEC-Foxn1^{eGFP+}-derived *Clono*TECs were purified by cell sorting and aggregated with cells from dGuo-treated E14.5 thymic lobes. RTOCs were transplanted into the kidney capsule of WT mice, ectopic thymi were recovered 4 weeks post-transplantation and analyzed by fluorescent microscopy. **(F and G)** Immunofluorescence analysis of ectopic thymus. Control and Foxn1^{eGFP} *Clono*TECs RTOCs were screened for GFP cells (anti-GFP Ab) and analyzed for the expression of EpCAM, MHCII and UEA as depicted. Square designates the zoomed area in (C). 50μm scale is shown. Images are representative of 5 ectopic thymus containing *Clono*TECs^{GFP}-derived cells.

Supporting information Figure 4



Supporting Information Figure 4. Clonogenic activity of cTECs decreases with time. (A) Age-matched postnatal day 5 (P5) or **(B)** mismatched P5 and 2-weeks-old cTECs from Actin^{GFP} and Actin^{RFP} reporter mice were co-cultured in a ratio 1:1 in midscale clonogenic assays for 12 days. *Clono*TEC^{GFP} and *Clono*TEC^{RFP} were analyzed by fluorescence microscopy and flow cytometry. The percentages of recovered cells analyzed by flow cytometry are represented in the graphs. Images represent complete individual wells and are illustrative of three experiments.