Chapter 2: Materials and Methods

2.1. Mice

2.1.A. Animal Facility

Mice were housed at the animal facility at the MRC Centre for Regenerative Medicine (CRM), University of Edinburgh. Wild type C57BL/6J mice were originally obtained from the Jackson Laboratory and maintained at CRM. Ly5.1 homozygous mice (CD45.1 homozygous) were also maintained by the CRM animal facility on a C57BL/6J background. Three transgenic strains were used in this thesis: iFoxn1 (Bredenkamp *et al.*, 2014), Rosa26CreERt2 (Hameyer *et al.*, 2007) and Foxn1^{G} (O'neill *et al.*, 2016). The *Foxn1*^{G} allele is a hypomorphic allele of Foxn1 and when in a homozygous state, *Foxn1*$^{G/G}$ mice display a nude athymic phenotype (data not published). These mice were used to generate C57BL/6J nude athymic mice for transplantation. All mice used were syngeneic to iTEC. Animal work was carried out in accordance with UK Home Office guidelines, as established in the Animals (Scientific Procedures) Act 1986.

2.1.B. Timed Matings

For timed matings, mice were placed in the same cages overnight. Embryos from females with a vaginal plug found the next morning were classified as embryonic day 0.5 (E0.5). WT embryos were the result of C57BL/6J x C57BL/6J crosses. iFoxn1 MEFs were established by crossing Rosa26CreERt2 males with iFoxn1 females.

2.1.C. 3R Considerations

The principles for the 3Rs (Replacement, Reduction and Refinement) were implemented during experimental design with use of mice. Use of mice was replaced with *in vitro* cocultures when possible. The number of mice used for experiments was reduced by sharing resources with other members of the lab and research groups. Refinement was implemented through ensuring animals were housed appropriately, and recommended anaesthesia, analgesia and post-operative care was administered according to veterinary advice to minimise any animal suffering and distress.

2.1.D. Sub-renal capsule transplantation

Four- to eight-week-old mice were anaesthetised with isoflurane gas and injected subcutaneously with RIMADYL® analgesic solution. A 2-3 cm dorsal midline incision was made using blunt tip scissors and the underlying dermis was separated from the body wall. A further 1 cm incision was made in the body wall parallel to the spine, above the kidney, taking care to avoid major vessels and spinal nerves. The kidney was exteriorized by applying gentle pressure and sterile saline was used frequently to prevent the kidney capsule from drying out. Curved fine forceps were used to lift the kidney capsule from the parenchyma and a small 2-4 mm incision was made with a needle. A glass Pasteur pipette that had been drawn out and fire-polished with a rounded closed end was dampened with saline and placed under the capsule tangential to the surface of the kidney to create a small pocket for placement of RTOC. The RTOC were inserted into the pocket generated, with fine forceps, and pushed away from the incision so that the grafts were located closest to the adrenal gland. Several RTOC could be placed evenly under the kidney capsule. The location of grafts was marked by inserting a small piece of isopore disc membrane (Merck Millipore Corporation, Ref# ATTP01300). The kidney was gently placed back in the body cavity and the muscle wall was sutured using VICRYL sutures 6-0 (Ethicon). The outer skin was closed using 2-3 surgical wound clips and the mice were administered 500 μl of saline subcutaneously to allow for rehydration. Wound clips were removed 7-10 days after surgery.

2.1.E. Isolation of Genomic DNA

Unique identification numbers were given to each transgenic mouse with ear clips. The ear sample collected for each mouse was used to obtain genomic DNA for genotyping. When using embryos, their heads were collected and processed in the same manner. The tissue lysis buffer used is shown in Table 2.1. Proteinase K (Promega) was added to the tissue lysis buffer at a concentration of 400 μg/ml. For ear samples 50 μl of the solution was used and for embryo heads 200 μl was used. Tissue digestion took place overnight in a shaking water bath at 56 ^{o}C for approximately 16 hours. The reaction was heat-inactivated at 95 ^{o}C for 10 mins. Samples were then cooled and centrifuged for 10 mins at 12,000 rpm at room temperature (RT) and stored at 4 ^{o}C.

Tissue Lysis Buffer	
Reagent	**Concentration**
Tris-HCl pH 8.3 (Roche)	10 mM
Magnesium Chloride ($MgCl_2$)	2.5 mM
Potassium Chloride (KCl)	50 mM
Gelatin	0.1 mg/ml
NP40	0.45%
Tween 20	0.45%

Table 2.1. Tissue lysis buffer reagents and concentrations.

2.2. Genotyping

All genotyping reactions used Taq polymerase and Qiagen associated buffers. PCR products were run on 1.5% agarose gels containing SYBR Safe DNA Gel Stain (Life Technologies) at a dilution of 1:1000. Prior to loading the gels 5 µl of Orange G dye (Sigma Aldrich) was added to each 30 µl sample and 10 µl of that was loaded into the gel. Gel electrophoresis was carried out at 100 V for approximately 25 mins.

Rosa26CreERt2 reaction		
Master Mix		
Reagent	Volume / reaction	
10 x buffer	3 µl	
10 mM dNTPs	0.5 µl	
Taq Polymerase	0.5 µl	
Forward primer 3	1 µl	
Reverse primer 4	1 µl	
H_2O	23.5 µl	
DNA	0.5 µl	
Thermocycler Programme		
Temperature	**Time**	
94 ºC	5 mins	
94 ºC	30 sec	30 cycles
58 ºC	30 sec	
72 ºC	1 min	
72 ºC	7 mins	
4 ºC	Infinite hold	
Band size: 269 base pairs		

Table 2.2. Reagents and thermocycler conditions for Rosa26CreERt2 transgene PCR amplification.

iFoxn1 reaction	
Master Mix	
Reagent	**Volume/reaction**
10 x buffer	3 μl
Q buffer	6 μl
10 mM dNTPs	0.5 μl
Taq Polymerase	0.5 μl
Forward primer 719	1 μl
Reverse primer 720	1 μl
H_2O	23.5 μl
DNA	0.5 μl
Thermocycler Programme	
Temperature	**Time**
94 °C	5 mins
94 °C	30 sec
60 °C	30 sec 35 cycles
72 °C	1 min
72 °C	10 mins
4 °C	Infinite hold
Band size: 750 base pairs	

Table 2.3. Reagents and thermocycler conditions for iFoxn1 transgene PCR amplification.

Foxn1^G reaction	
Master Mix	
Reagent	Volume / reaction
10 x buffer	3 µl
Q buffer	6 µl
10 mM dNTPs	0.5 µl
Taq Polymerase	0.5 µl
Forward primer 260	1 µl
Reverse primer 261	1 µl
H_2O	23.5 µl
DNA	1 µl
Thermocycler Programme	
Temperature	**Time**
94 °C	5 mins
94 °C	30 sec 30 cycles
57 °C	45 sec
72 °C	1 min
72 °C	10 mins
4 °C	Infinite hold
Band size: 220 base pairs	

Table 2.4. Reagents and thermocycler conditions for Foxn1^G transgene PCR amplification.

2.3. Tissue collection and processing

2.3.A. Embryo isolation and dissection

Pregnant female mice were culled by cervical dislocation at the desired gestational day and embryos were removed from the uterus and placed in PBS on ice. A stereomicroscope was used to remove embryos from the yolk sack and perform decapitation. An incision was made below the sternum and extended all the way up to the neck to reveal the thoracic cavity. Fetal thymic lobes were located by following the left and right common carotid arteries upwards from the heart. Fetal thymic lobes at E13.5 can be located near the third pharyngeal pouch and E15.5 lobes are located directly above the heart. Fetal thymic lobes were collected from each embryo. Tissue surrounding thymic lobes was removed and placed in PBS at 4 ^{o}C until digestion. Thymic lobes were enzymatically digested through incubation in TrypLE Express solution (Life Technologies, Ref# 12604013) for 5 minutes at 37 ^{o}C in a thermomixer set to 1400 rpm. Following this, mechanical dissociation was applied by triturating with a P1000 ten times and the reaction was inactivated with chilled 2% FCS / PBS buffer (2% FACS buffer). Cell suspensions were filtered through a 70 μm cell strainer (Fisher Scientific UK Ltd, Ref# 10788201) and centrifuged at 500 relative centrifugal force (RCF) for 5 minutes. Fetal thymic cells were processed in 2% FACS buffer with DNAse (0.05 mg/ml) (Lorne Laboratory).

2.3.B. Mouse Embryonic Fibroblast Establishment

Mouse Embryonic Fibroblasts (MEFs) were established from E13.5 embryos. Embryos were collected, decapitated (used for genomic DNA isolation), lower limbs and tail removed, and internal organs stripped. The remaining tissue was homogenized with an 18G needle and enzymatically digested for 10 minutes at 37 ^{o}C in 0.25% trypsin EDTA (Invitrogen). Trypsin was inactivated with iTEC medium containing serum (section 2.8.A.I) and further mechanically dissociated by syringing up and down eight times with a 23G needle and syringe. Cell suspensions were centrifuged at 300 RCF for 5 mins and resuspended in iTEC medium. Cells were seeded into a gelatinised T75 Corning flask (Corning #CLS430641U) with 12 ml of iTEC medium. Cells were passaged 1:3 at 100% confluency.

2.3.C. Adult thymus thymocytes isolation

To obtain thymocytes from the adult thymus, four to six-week-old mice were culled using cervical dislocation. The rib cage and sternum were removed to expose the thoracic cavity and obtain the thymus. To release T cells, the thymus was compressed through a 40 μm cell strainer (Fisherbrand, Ref#11587522) with a syringe plunger. Processing took place in 2% FACS buffer (2% FCS in PBS) with 2 mM EDTA (AccuGENE, Ref# 51234) at 4 ºC.

2.3.D. Graft thymocyte isolation

To obtain thymocytes from recovered grafts, transplant recipients were culled using cervical dislocation and their transplanted kidney was removed. Grafts were located under the kidney capsule and removed by incising the surrounding area with a fine needle and forceps. The graft was compressed through a 40 μm cell strainer with a syringe plunger and processed for flow cytometric analysis in 2% FACS buffer with 2 mM EDTA at 4 ºC.

2.3.E. Peripheral blood collection

Peripheral blood samples were collected from *Foxn1*$^{G/G}$ transplant recipients to assess circulating T cell populations. Blood samples were collected via tail vein sampling whereby a small incision was made with a scalpel and less than 100 μl of blood was collected in Sarstedt Microvette CB 300 tubes (Starstedt Ltd, Ref# 16.444.100) via capillary action. T cell numbers calculated during flow cytometry analysis were adjusted to reflect the volume of blood analysed from each recipient and normalized to reflect total numbers found in 1 ml of peripheral blood.

2.3.F. Lymph node and spleen thymocyte isolation

Foxn1$^{G/G}$ transplant recipients were culled at the experimental endpoint with cervical dislocation and skin and fascia were exposed to reveal axillary and inguinal lymph nodes. Three cervical lymph nodes associated with the salivary glands were also collected from each recipient to give a total of seven lymph nodes. Axillary, inguinal and cervical lymph nodes were pooled for each recipient and also compressed through a 40 μm cell strainer and processed for flow cytometry in 2% FACS buffer with 2 mM EDTA at 4 ºC. Transplant recipient spleens were also collected and thymocytes isolated in the same manner. Following spleen compression, isolated cells were pelleted at 300 RCF for 5 minutes and the supernatant was aspirated. 10X Red Blood Cell (RBC) Lysis Buffer (BioLegend, Ref#420301) was diluted to a 1X working concentration with deionized water and cell pellets were resuspended in 5 ml of the 1X RBC Lysis buffer. Samples were incubated for 5 minutes at 4 ºC. Samples were centrifuged at 300 RCF at 4 ºC and resuspended in 2% FACS buffer with 2 mM EDTA for processing. T cells numbers calculated during flow cytometry were corrected to reflect total number of T cells present in seven lymph nodes or one whole spleen.

2.3.G. Magnetic enrichment of CD45$^+$ cells from spleen and lymph nodes of transplanted host for TCRβ-Sequencing

The experimental endpoint of some of the *Foxn1$^{G/G}$* iTEC transplantation experiments occurred during the Covid-19 pandemic lockdown and FACS facilities were not in use. In order to collect lymph node and spleen thymocytes for TCR Repertoire Diversity RNA-Sequencing, CD45$^+$ enrichment took place using magnetically labelled cells. Cells were stained with antiCD45-magnetic bead conjugates (Miltenyi Biotec, Ref# 130-052-301) at 4 ^{o}C for 15 minutes. Cells were washed twice with centrifugation and resuspension in 2% FACS buffer to remove unbound antibody. Separation of CD45 magnetically labelled cells was achieved with QuadroMACs Separator and LS columns (Miltenyi Biotec). The LS columns were washed with 3 ml of 2% FACS buffer before addition of labelled cells. The columns were washed an additional three times to remove any unbound cells. To collect the CD45$^+$ cell fraction, the LS columns were removed from the magnet and plunged out the column. A cell count was performed using a TC20^{TM} Automated Cell Counter (BioRad) and 1 x 10^6 cells / sample were aliquoted into an Eppendorf microcentrifuge tube, centrifuged at 500 RCF for 5 minutes and the supernatant discarded. Cell pellets were snap frozen on dry ice and stored at - 80 ^{o}C until further processing.

2.3.H. Magnetic enrichment of CD117$^+$ (c-KIT) fetal liver T cells

To obtain lymphoid primed multipotent progenitors (LMPPs) from E15.5 fetal livers, splenocytes were magnetically enriched for cells expressing c-KIT in the same manner as above but with the use of antiCD117-magnetic bead conjugates (Miltenyi Biotec, Ref# 130-091-224). The CD117$^+$ fraction was collected and further stained for FACS sorting.

2.4. Flow cytometry

2.4.A. Antibody staining protocol

Directly conjugated antibodies were used for flow cytometry sorting and analysis. Antibodies were all titrated and optimized when designing panels. For surface antigen, cells were stained in 2% FACS buffer with 2 mM EDTA with the appropriate concentration of selected antibodies and incubated for 15 minutes at 4 ^{o}C unless stated otherwise. In cases where multiple Brilliant Violet^{TM} antibodies were used cells were stained in Brilliant Stain Buffer (BD Pharmingen, Ref# 563794) to eliminate non-specific reactivity between polymers, as recommended by the manufacturer. For some dim antibodies cells were stained for 30 minutes at room temperature (RT) prior to addition of the remaining antibodies. Cells were then washed twice with 2% FACS buffer by centrifugation at 300 RCF for 5 minutes and resuspended. 4’,6-diamidino-2-phenylindole (DAPI) or 7-aminoactinomycin D (7-AAD) membrane permeable nuclear dyes were used to discriminate between live and dead cells depending on the chosen panel. Addition of these took place immediately before sorting or analysis. In panels where intracellular antigen staining took place and required fixation, following surface antigen staining, cells were washed twice with PBS to remove excess antibodies and serum and stained with Fixable Viability Dye eFluor450 (Life Technologies, Ref# 65-0863-14) or Zombie Green^{TM} Fixable Viability kit (BioLegend, Ref# 423111) for 30 minutes on ice. Cells were then washed once with PBS and 1 ml of Fixation/Permeabilization working solution (one part Foxp3 Fixation/Permeabilization Concentrate with three parts Foxp3 Fixation/Permeabilization Diluent) was added to each tube and pulse vortexed. Samples were incubated for 30-60 minutes at RT or overnight at 4 ^{o}C. 2 ml of 1X Permeabilization Buffer (one part 10X Permeabilization Buffer with nine parts distilled water) was added to each tube and samples were centrifuged at 500 RCF for 5 minutes and the supernatant discarded. Cell pellets were resuspended in residual volume of 1X Permeabilization Buffer (~100 µl), and the appropriate concentration of antibody directly added and incubated for 30 minutes at RT. Samples were washed twice with 2 ml of 1X Permeabilization buffer and resuspended in 200 µl of PBS for analysis by flow cytometer.

2.4.B. Flow cytometry sorting and analysis

FACS sorting was performed using a BD FACSAriaII^{TM} or Fusion cell sorter with Diva 4.1 software (BD Biosciences) and performed by the Centre for Regenerative Medicine’s FACS facility staff. Flow cytometry analysis was carried out using a BD 5LSRFortessa^{TM} (BD Biosciences) or a NovoCyte with NovoExpress 1.3.0. software (ACEA). All analysis was performed with FCS Express 7 (De Novo Software).

2.5. Histology

2.5.A. Tissue Fixation and sectioning

Recovered kidneys with grafts or adult thymi were fixed in 4% paraformaldehyde (PFA) overnight at 4 ºC on an agitator. Samples were then washed three times in PBS for 5 minutes at RT and grafts were removed from under the kidney capsule and equilibrated in 15% sucrose in PBS overnight at 4 ºC, until samples sunk to the bottom of the tube. Samples were then embedded in 15% sucrose/ 7% gelatin in PBS solution at 37 ºC for 1 hour and transferred to a bare mold and filled with fresh pre-warmed sucrose/gelatin. Samples were snap frozen in liquid N_2 (LN_2) and stored at -80 ºC for at least 24 hours. Cryo-sectioning took place at -24 ºC and 7-10 µm sections were transferred to PoLysine® slides (VWR) and stored at -80 ºC until staining. Slides were thawed at RT prior to staining and gelatin was dissolved from samples by microwaving for 45 seconds in PBS.

2.5.B. Haematoxylin and eosin staining

For hematoxylin and eosin (H&E) staining sections were placed in haematoxylin (Thermo Fisher, Ref# 008011) for 5 minutes followed by rinsing in running tap water for 3 minutes. Slides were places in 1% acid alcohol for 5-10 seconds, rinsed in tap water and placed in Scotts tap water for 25 seconds, followed by another rinse in water for 3 minutes. Eosin (Thermo Fisher, Ref# 676-6018) staining took place for 25 seconds and samples were again rinsed in running tap water and transferred to 70% ethanol (EtOH), 80% EtOH, 95% EtOH each for 20 seconds and lastly in 100% EtOH twice for 20 seconds. Finally, samples were placed in xylene solution twice for 5 minutes and mounted in pertex (Histolab, Ref# 00801-EX) with glass coverslips #1.5 (VWR, Ref# 631-0138) and stored at RT until imaging.

2.5.C. Immunohistochemistry staining

Sections were circled with a hydrophobic pen and allowed to dry. Sections were blocked in 5% goat serum in 0.1% tween 20/PBS (PBST) solution for 30 minutes at RT. Blocking solutions were tapped off and primary antibody at the appropriate concentration was added in 1% goat serum/ PBST for 2 hours at RT or overnight at 4 ºC in a humidity chamber. Slides were washed three times for 5 minutes in PBST. Secondary antibodies were centrifuged at 2×10^4 RCF for 10 minutes to remove antibody aggregated and diluted in 1% goat serum/PBS and added for 1 hour at RT or overnight at 4 ºC in the dark. Slides were washed three times for 5 minutes in PBST. In cases where the cell nucleus required visualisation, DAPI was used at a concentration of 5 µg/ml for 2 minutes. Slides were washed twice for 5 minutes in PBST followed by a final wash in distilled H_2O and allowed to dry prior to mounting with Vectashield Hardset Antifade Mounting Medium (Vector Laboratories) and a glass coverslip #1.5 (VWR, Ref# 631-0138). Slides were stored at 4 ^{o}C prior to imaging.

2.5.D. Imaging

Wholemount kidney images were taken on a Leica M165 FC stereomicroscope. For H&E a ZEISS Axio Observer widefield inverted microscope was used. For IHC either the ZEISS Axio Observer or a Leica TCS SP8 confocal microscope with 5 detectors was used. All post-acquisition analysis was performed on ZEN 2.3 (blue edition) ZEISS software or on ImageJ v1.53c.

2.6. Gene expression analysis

2.6.A. Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

2.6.A.I. cDNA synthesis and amplification

100 cell bulk cell populations were FACS sorted into PCR tubes with 10 µl CellsDirect 2X Reaction Mix (CellsDirect, Invitrogen) and 0.2 ul SUPERase-In RNase inhibitor (Ambion), centrifuged briefly and snap frozen on dry ice. Samples were stored at -80 ^{o}C prior to use. Gene-specific reverse transcription of mRNA was utilized to create cDNA. Forward and reverse primers for each gene were mixed at 20 µM concentration each and 1 µl of each gene of interest mix was combined with water to make a 100 µl solution (4 x Assay mix). A preamplification mix was generated (Table 2.5) and 10 µl of it added to each sample followed by thermal cycling (Table 2.6).

Preamplification mix	
4 x Assay Mix	5 µl
Superscript III/Taq mix (Cells direct)	1 µl
DNase, RNase free water	4 µl

Table 2.5. Preamplification mix for cDNA synthesis.

Thermal cycling programme		
Temperature	**Time**	**Cycles**
50 ºC	15 mins	1 cycle
95 ºC	2 mins	18 cycles
95 ºC	15 sec	
60 ºC	4 mins	1 cycle
4 ºC	∞	

Table 2.6. Thermal cycling conditions for cDNA synthesis.

2.6.A.II. Real time fluorescence detection

20 µl of prepared cDNA was diluted in 280 µl DNase, RNase free water. A RT-qPCR gene-specific mastermix solution was prepared for each gene of interest (Table 2.7). A 384-well plate (Roche) was loaded with 5.325 µl of RT-qPCR gene-specific mastermix per well and 4.583 µl of diluted cDNA in triplicate. Plates were sealed and centrifuged at 300 RCF for 1 minute prior to loading into a Lightcycler 480-II (Roche) to perform real time fluorescence detection (Table 2.8).

RT-qPCR gene-specific mastermix	
Reagent	Volume / well
2 x Master mix (Roche)	5 µl
UPL probe (Roche)	0.1 µl
20 µM forward and reverse primer mix (Sigma)	0.225 µl

Table 2.7. RT-qPCR gene-specific mastermix.

Thermal cycling programme		
Temperature	**Time**	**Cycles**
95 °C	5 mins	1 cycle
95 °C	10 sec	45 cycles
60 °C (Signal detection)	20 sec	
40 °C	10 sec	1 cycle

Table 2.8. Thermal cycling conditions for RT-qPCR.

2.6.A.III. Relative gene expression quantification

Relative expression levels were determined using the Roche Universal Probe Library on the Roche Lightcycler 480 after normalisation to the geometric mean of three housekeeping genes (*Hmbs, Hprt* and *Tbp*) and using the comparative Ct method ($2^{-\Delta\Delta Ct}$ method) as described by Livak and Schmittgen (Livak and Schmittgen, 2001).

2.7. RNA Sequencing

2.7.A. SmartSeq2 RNA-sequencing of WT MEF and FTM bulk cell populations

100 cells of each cell population were sorted into 4 μl of SmartSeq2 cell lysis buffer in PCR tubes. The cell lysis buffer consisted of 0.4% v/v Triton X-100 (Sigma-Aldrich, Ref# T9284), 2 U/μl RNase inhibitor (Clonetech, Ref# 2313A), 2.5 mM dNTPs (Thermo-Fisher, Ref# R0192), 2.5 μM Oligo-dT30VN (5′–AAGCAGTGGTATCAACGCAGAGTACT30VN-3′) and 4 x 10^7 dilution of ERCC spike in (Thermo Fisher, Ref# 4456740). Nine samples were collected for each cell population (WT MEFs and Fetal Thymic Mesenchyme) with three being collected for each of the three independent experiments. RNA-Sequencing was performed at the MRC Weatherhall Institute of Molecular Medicine and the University of Oxford as described (Sheridan *et al.*, 2009).

2.7.B. V-region TCR RNA-Sequencing of peripheral T cells in grafted mice

1 x 10^6 magnetically enriched $CD45^+$ spleen or lymph node cells were collected for each grafted recipient at 22 weeks post-transplantation (except animal ID 315:20 weeks, ID 501: 17 weeks) and cell pellets were snap frozen on dry ice and stored at -80 °C. A total of 34 cDNA libraries were prepared in randomised order of 6 samples at a time to avoid batch effects. Lysis buffer, dynabeads and wash buffer A and B were part of the Dynabeads mRNA Purification DIRECT Kit (ThermoFisher Scientific, Ref#61012). 300 μl of lysis buffer was added to each sample and mixed to obtain complete lysis. Dynabeads were resuspended by gently shaking and 100 μl were transferred to an RNase-free 1.5 ml microcentrifuge tube and placed on a magnet. After 30 seconds the supernatant was removed, and beads were washed with 50 μl lysis/binding buffer off the magnet. The tubes were placed back on the magnet for 30 seconds and the supernatant was removed. Sample lysates were added to the beads, mixed and incubated for 5 minutes at RT followed by placement on the magnet for 2 minutes and the supernatant was removed. Beads were

washed with 600 µl wash buffer A at RT and placed on the magnet. Wash buffer A was removed, and beads were washed twice with 300 µl of wash buffer B at RT using the magnet to separate beads from the solution. Beads were resuspended in 9 µl of water, transferred to PCR tubes with 11 µl of cDNA mastermix (table 2.9) and placed in a pre-warmed PCR machine at 42 ^{o}C for 45 minutes. Samples were manually mixed every 15 minutes. cDNA synthesis reactions were terminated by heating at 70 ^{o}C for 10 minutes and then placed on ice. 1 µl of Uracyl DNA glyocosylase (5U/ µl) (New England BioLabs, Ref# M0280S) was added to each sample and incubated at 37 ^{o}C for 15 minutes. Amplification mix for TCRβ V variable regions were prepared for each sample (table 2.10). Each sample received a unique P7-SMARTamp(index) primer to allow for demultiplexing following sequencing. Thermocycling was carried out as described in Table 2.11. Amplicons were run on a 2% agarose (Ultrapure agarose) gels. TCR amplicons were pooled and 450-650 bp bands were extracted and purified using the Monarch Gel Extraction kit (New England BioLabs, Ref# T1020L) according to manufacturers recommended instructions. MiSeq 2 x 300 bp with custom primers RNA-Sequencing was carried out by GENEWIZ®. This protocol was optimized and provided by the Cowan Lab at the University of Edinburgh and was carried out in collaboration with them.

cDNA Mastermix	
Reagent	**Volume / sample**
First Strand Buffer (Clontech, Ref# 639537)	4 µl
DTT (Clontech, Ref# 639537)	0.5 µl
dNTP mix 10 mM (ThermoFisher Scientific, Ref# R0192)	2 µl
SMARTNNN oligo [10 µM] (Integrated DNA Technologies)	2 µl
RNAse inhibitor (Clontech, Ref# 2313A)	0.5 µl
SMARTScribe RTase (Clontech, Ref# 639537)	2 µl

Table 2.9. cDNA synthesis mastermix.

Amplification Mix	
Reagent	**Volume / sample**
Phusion high-fidelity PCR mastermix (ThermoFisher Scientific, Ref# F548S)	10
P5-mTCRBrev3 [12 μM] (Integrated DNA Technologies)	2
P7-SMARTamp(index) [10 μM] (Integrated DNA Technologies)	2
dH2O (ThermoFisher Scientific, Ref# AM9938)	4
cDNA	2

Table 2.10. Amplification mix used to amplify TCRβ V variable gene regions.

Thermocycling conditions		
Temperature	**Time**	**Cycles**
98	2 mins	1 cycle
98	5 sec	5 cycles
60	10 sec	
72	15 sec	
98	5 sec	25 cycles
65	10 sec	
72	25 sec	
72	2 mins	1 cycle

Table 2.11. Thermocycling conditions used to amplify TCRβ V variable gene regions.

2.7.D. Bioinformatics analysis of RNA-Sequencing datasets

Bioinformatics analysis for SmartSeq2 datasets was carried out by Dr. Paul Rouse using Linux and R studio. Bioinformatics analysis for TCR repertoire diversity was carried out by Natasha Smith and Dr. Graeme Cowan (University of Edinburgh).

2.7.D.I. Bioinformatic analysis of SmartSeq2 bulk RNA-Seq data

FASTQ files were imported to Linux and quality control tested. Datasets were trimmed of adapter sequences with the read trimming tool: Trimmomatic. Reads were aligned to the reference genome Mus_musculus.RGCm38 with STAR and data was loaded into R studio to generate count matrixes with the Rsubread tool. Count matrixes, with rows represented genes and columns representing samples, for all three datasets (FTM, WT MEF, TEC) were merged and annotated using the BioMart-Ensembl tool. Normalisation was carried out using DESeq2 and a new table was generated. Ramilowski pairs were identified and the human2mouse tool from the homologene package applied. A new table was generated whereby counts of ligands (on FTM or WT MEFs) multiplied by count of corresponding receptor (on TEC) were generated. Datasets failed to pass the Shapiro-Wilks normality test, so statistical significance between $\text{FTM}^{\text{ligand}}$ x $\text{TEC}^{\text{receptor}}$ and WT $\text{MEF}^{\text{ligand}}$ x $\text{TEC}^{\text{receptor}}$ was carried out using a Mann-Whitney test. Detailed code can be found in the appendix (Appendix, 8.2)

2.7.D.II. Bioinformatics analysis for TCR repertoire diversity

Bulk TCR sequence data was processed using MiGEC software. A TCR clone was defined by 100% amino acid sequence identity of the CDR3 region and IMGT nomenclature used for gene usage. Only in-frame CDR3s were analysed. Detailed code can be found in the appendix (Appendix, 8.3).

2.8. Tissue culture

2.8.A. Tissue culture medium

2.8.A.I. iTEC Medium

- Advanced DMEM/F12 (Life Technologies, Ref# 12634010)
- 10% FCS (Life Technologies)
- 1% penicillin (10,000 units/ml) / streptomycin (10,000 µg/ml) (Invitrogen, Ref# 15140-122)
- 1% GlutaMax supplement (Life Technologies, Ref# 35050061)
- 1% non-essential amino acids (NEAA) (Invitrogen, Ref# 11140-036)

2.8.A.II. 2% iTEC Medium

- Advanced DMEM/F12 (Life Technologies, Ref# 12634010)
- 2% FCS (Life Technologies)
- 1% penicillin (10,000 units/ml) / streptomycin (10,000 µg/ml) (Invitrogen, REF# 15140-122)
- 1% GlutaMax supplement (Life Technologies, Ref# 35050061)
- 1% non-essential amino acids (NEAA) (Invitrogen, Ref# 11140-036)

2.8.B. Murine Embryonic Fibroblast culture

MEFs established from E13.5 embryos growing in T75 flasks in iTEC medium reached confluency within 24 hours. To passage MEFs, cells were washed twice with 10 ml of PBS and 1 ml of pre-warmed 0.25% trypsin EDTA was added and incubated at 37 ^{o}C for 2 minutes. The reaction was inactivated with iTEC medium and cells were passaged into a T150 (Corning, Ref# CLS430825) with 15 ml of iTEC medium. Once reaching confluency cells were passaged 1:3 by washing cells in 10 ml of PBS before adding 3 ml 0.25% trypsin EDTA for 1 minute at 37 ^{o}C and the above process was repeated. The passaging process was repeated once more prior to cryopreservation. For cryopreservation, each T150 was split into three cryovials and frozen down in iTEC medium supplemented with 10% DMSO. Cryovials were transferred to -80 ^{o}C overnight in a Mr Frosty propan-2-ol container (Nalgene ThermoFischer) and transferred to LN_2 after 24 hours.

2.8.C. iFoxn1 MEF reprogramming into iTEC

iFoxn1 MEF cryovials were thawed in a 37 ^{o}C water bath for 2 minutes. Cells were washed in 10 ml iTEC medium, centrifuged at 300 RCF for 3 minutes and resuspended in 25 ml iTEC medium and seeded into a 0.1% gelatin coated T150 flask. Following 48-hour culture, cells were passaged into three T150 flasks and left to adhere to the flask for four hours. After four hours, cells were washed once with PBS and the medium was replaced with 30 ml iTEC medium supplemented with 1.6 µM 4-hydroxy tamoxifen (4-OHT, Merck Chemicals Ltd, Ref# 5082250001) to induce Cre-mediated recombination. Medium was replaced completely every second day. iTEC were collected at either 4 or 18 days post 4-OHT. To harvest iTEC, T150s were washed twice with PBS and incubated in 3 ml of 0.25% trypsin EDTA at 37 ^{o}C for 5 minutes.

2.8.D. Reaggregated Thymic Organ Culture

Reaggregated thymic organ cultures were generated as described (Sheridan *et al.*, 2009). Single cell suspensions were drawn into a parafilm sealed non-bevelled pipette tip. The cell suspensions were reaggregated by compaction reaggregation via centrifugation at 500 RCF for 5 minutes. Compacted cell pellets were extruded at the liquid-gas interface onto a polycarbonate filter paper raft (Merck Millipore, Ref# ATTP01300) floating on 1ml of iTEC medium in 24 well plates. Reaggregation took place over the course of 18 or 60 hours prior to transplantation.

2.8.E. FTM candidate supplementation

In cases where RTOC were cultured longer than 60 hours *in vitro,* 50% medium changes were employed. Candidate soluble factors were supplemented in 2 % iTEC medium at 5 ng/ml each. At 50% medium changes 500 µl of media was removed from the 24-well plate and replaced with 2X candidate solutions in 2% iTEC medium. All candidates were purchased from PEPROTECH®.

2.9. Antibody Panels

Described are antibody combinations used for flow cytometry and immunohistochemistry.

2.9.A. FACS Panels

2.9.A.I. iTEC isolation from iFoxn1 MEFs

Antigen	**Fluorophore**	**Dilution**	**Supplier**	**Catalogue Number**	**Clone**
DLL4	PE	1:1600	BioLegend	130808	HMD4-1
DAPI	-	0.5 µg/ml	Life Technologies	D1306	-

Table 2.12. Antibody panel used to isolate iTEC from iFoxn1 MEFs at day 4 or 18 post tamoxifen addition.

2.9.A.II. Fetal Thymic Mesenchyme and DN Thymocyte isolation

Antigen	Fluorophore	Dilution	Supplier	Catalogue Number	Clone
Lineage:					
CD3ε	FITC	1:1000	BioLegend	100305	145-2C11
CD4	FITC	1:1000	BioLegend	100509	RM4-5
CD8	FITC	1:1000	BioLegend	100705	53-6.7
CD11b	FITC	1:1000	eBioscience	11-0112-82	M1/70
CD11c	FITC	1:1000	eBioscience	11-0114-82	N418
B220	FITC	1:1000	BioLegend	103205	RA3-6B2
GR-1	FITC	1:1000	BioLegend	108405	RB6-8C5
NK1.1	FITC	1:1000	BioLegend	108705	PK136
TER119	FITC	1:1000	BioLegend	116205	TER-119
CD45	PerCP-Cy5.5	1:1000	eBioscience	45-0451-82	30-F11
PDGFRα	APC	1:1000	BioLegend	135907	APA5
PDGFRβ	APC	1:1000	BioLegend	136007	APB5
DAPI	-	0.5 μg/ml	Life Technologies	D1306	-

Table 2.13. Antibody panel used to isolate fetal thymic mesenchyme and DN thymocytes from E15.5 thymic lobes.

2.9.A.III. Thymic Epithelial Progenitor Cell (TEPC) isolation

Antigen	Fluorophore	Dilution	Supplier	Catalogue Number	Clone
EpCAM	PE	1:1000	BioLegend	324205	G8.8
PLET1	Alexa Fluor^{TM} 647	1:500	-/Life Technologies	A21247	-
Lineage:					
CD3ε	FITC	1:1000	BioLegend	100305	145-2C11
CD4	FITC	1:1000	BioLegend	100509	RM4-5
CD8	FITC	1:1000	BioLegend	100705	53-6.7
CD11b	FITC	1:1000	eBioscience	11-0112-82	M1/70
CD11c	FITC	1:1000	eBioscience	11-0114-82	N418
B220	FITC	1:1000	BioLegend	103205	RA3-6B2
GR-1	FITC	1:1000	BioLegend	108405	RB6-8C5
NK1.1	FITC	1:1000	BioLegend	108705	PK136
TER119	FITC	1:1000	BioLegend	116205	TER-119
DAPI	-	0.5 µg/ml	Life Technologies	D1306	-

Table 2.14. Antibody panel used to isolate E13.5 TEPC. TEPC were used as a positive control for RT-qPCR experiments.

2.9.A.IV. Thymic Epithelial Cells and Fetal Thymic Mesenchyme isolation

Antigen	**Fluorophore**	**Dilution**	**Supplier**	**Catalogue Number**	**Clone**
EpCAM	FITC	1:1000	BioLegend	118207	G8.8
PDGFRα PDGFRβ	APC APC	1:2000 1:2000	BioLegend BioLegend	135907 136007	APA5 APB5
TER119	PE	1:3000	BioLegend	116207	TER-119
CD31	PE-Cy7	1:1600	BioLegend	102523	MEC 13.3
CD45	APC-Cy7	1:1000	BD Biosciences	557659	30-F11
DAPI	-	0.5 µg/ml	Life Technologies	D1306	-

Table 2.15. Antibody panel used to isolate E15.5 TEC and FTM.

2.9.A.V. Isolation of Lymphoid-primed Multipotent Progenitors (LMPPs)

Antigen	**Fluorophore**	**Dilution**	**Supplier**	**Catalogue Number**	**Clone**
Lineage:					
CD3	FITC	1:100	BioLegend	100306	145-2C11
CD5	FITC	1:1000	BioLegend	100605	53-7.3
CD19	FITC	1:500	BioLegend	152403	1D2
Gr-1	FITC	1:800	BioLegend	108405	RB6-8C5
B220	FITC	1:100	BioLegend	103205	RA3-6B2
TER119	FITC	1:1000	BioLegend	116205	TER-119
F4/80	FITC	1:1600	BioLegend	123108	2BM8
NK1.1	FITC	1:800	BD Bioscience	553164	PK136
CD135(Flt3)	APC	1:200	BioLegend	108107	A2F10
SCA-1	PE	1:2000	BioLegend	108107	D7
c-KIT (CD117)	PE-Cy7	1:1600	BioLegend	105813	2B8
DAPI	-	0.5 µg/ml	Life Technologies	D1306	-

Table 2.16. Antibody panel used to isolate LMPPs from E15.5 fetal liver.

2.9.A.VI. T cell lineage commitment panel for recovered grafts

Antigen	**Fluorophore**	**Dilution**	**Supplier**	**Catalogue Number**	**Clone**
CD3ε	PE	1:200	eBioscience	12-0031-82	145-2C11
TCRβ	PerCP-Cy5.5	1:200	BioLegend	109228	H57-597
CD4	PE-Cy7	1:800	eBioscience	25-0042082	RM4-5
CD8	APC	1:800	eBioscience	17-0081-82	53-6.7
CD45	AF700	1:200	BioLegend	103128	30-F11
FOXP3	AF488	1:200	BioLegend	126405	MF-14
Fixable viability dye	eFluor 450	1:1000	eBioscience	65-0863-14	-

Table 2.17. Antibody panel used to determine T cell lineage commitment in recovered iTEC grafts.

2.9.A.VII. Host vs donor T cell contribution panel for recovered grafts

Antigen	Fluorophore	Dilution	Supplier	Catalogue Number	Clone
CD45.1	FITC	1:2000	BioLegend	110706	A20
CD45.2	BV570	1:10	BioLegend	109833	104
CD4	BV650	1:200	BioLegend	100545	RM4-5
CD8	APC	1:1600	eBioscience	17-0081-82	53-6.7
CD3ε	BV785	1:100	BioLegend	100355	145-2C11
TCRβ	PE-Cy7	1:100	BioLegend	109222	H57-597
CD69	BV421	1:100	BioLegend	104528	H1.2F3
CD62L	APC-Fire 750	1:800	BioLegend	104450	MEL/14
CD44	BV510	1:1000	BioLegend	103030	IM7
CD25	PE	1:1000	BioLegend	102007	PC61
7-AAD	-	-	BioLegend	420403	-

Table 2.18. Antibody panel used to analyse T cell subsets from host and donor in grated mice.

2.9.A.VIII. Peripheral T cell panel for grafted mice

Antigen	**Fluorophore**	**Dilution**	**Supplier**	**Catalogue Number**	**Clone**
CD3ε *	BV785	1:100	BioLegend	100355	145-2C11
CD4	BV650	1:200	BioLegend	100545	RM4-5
CD8 *	BV570	1:50	BioLegend	100739	53-6.7
TCRγδ	BV605	1:400	BioLegend	118124	GL3
CD44 *	BV510	1:1000	BioLegend	103030	IM7
CD127	BV421	1:100	BioLegend	135024	A7R34
TCRβ	PE-Cy7	1:100	BioLegend	109222	H57-597
CD25 *	PerCP-Cy5.5	1:50	BioLegend	102029	PC61
Ki67	PE	1:100	eBioscience	12-5698-82	SolA15
CD62L	APC-Fire 750	1:800	BioLegend	104450	MEL/14
FOXP3	AF647	1:200	BioLegend	126408	MF-14
Zombie Green	-	1:1000	BioLegend	423111	-

Table 2.19. Antibody panel used to analyse T cell subsets in peripheral blood, lymph nodes and spleen of transplanted mice. Antibodies with an * were incubated for 30 minutes at RT prior to addition of the rest.

2.9.B. Immunohistochemistry antibodies

2.9.B.I. Primary antibodies used during immunohistochemistry

Antigen	Host	Dilution	Supplier	Catalogue Number	Clone
Cytokeratin 8 (K8)	Rabbit	1:250	Abcam	ab53280	EP1628Y
Biotinylated Ulex Europaeus Agglutinin I (UEA1)	-	1:500	Vector Laboratories	B-1065	-
β5t	Rabbit	1:100	MCL	PD021	PD021
MHCII	Rat	1:250	Abcam	Ab15630	ER-TR3
Cytokeratin 14 (K14)	Chicken	1:500	BioLegend	906004	Poly9060
Aire	Rat	1:100	eBioscience	14-5934-82	5H12
CD45	Rat	1:100	TONBO bioscience	70-0451-U100	30-F11
Vimentin	Rabbit	1:250	Abcam	ab92547	EPR3776

Table 2.20. Primary antibodies used for immunohistochemistry staining of tissue sections.

2.9.B.II. Secondary antibodies used for immunohistochemistry

Specificity	Fluorophore	Host	Dilution	Supplier	Catalogue Number
Rabbit	Alexa Fluor 488	Goat	1:500	ThermoFisher	A-11001
Chicken	Alexa Fluor 568	Goat	1:500	VWR	20104-1
Biotin	Streptavidin Alexa Fluor 647	-	1:500	ThermoFisher	S21374
Rat	Alexa Fluor 568	Goat	1:500	ThermoFisher	A-11031
Rat	Alexa Fluor 405	Goat	1:500	ABCAM	ab175671

Table 2.21. Secondary antibodies used for immunohistochemistry staining of tissue sections.

2.10. Primers

All DNA oligonucleotide primers were designed using the Universal Probe Library (Roche) software. Primers with the highest score were selected and ordered from Sigma. Primers used for TCRβ V-region sequencing were all purchased from Integrated DNA Technologies.

2.10.A. Genotyping primers

Transgene	**Direction**	**Sequence**
Rosa26CreERt2	Forward	GCATAACCAGTGAAACAGCATTGCTG
	Reverse	GGACATCAGGGATCGCCAGGCG
iFoxn1	Forward	GGGAGCAGCTGAAGGATGAC
	Reverse	CGCTTGAGGAGAGCCATTTG
Foxn1^G	Forward	TATATCATGGCCGACAAGCA
	Reverse	GAACTCCAGCAGGACCATGT

Table 2.22. Oligonucleotide sequences used to genotype transgenic mice.

2.10.B. RT-qPCR primers

Transgene	Direction	Sequence	UPL Probe Number
Endogenous Foxn1	Forward	CTTAAAGGTCAAAGAAGGAAAACACT	68
	Reverse	GGCTAACAAATAAGTTGGCTGA	
Total Foxn1	Forward	TGACGGAGCACTTCCCTTAC	94
	Reverse	GACAGGTTATGGCGAACAGAA	
Dll4	Forward	AGGTGCCACTTCGGTTACAC	106
	Reverse	GGGAGAGCAAATGGCTGATA	
Dll1	Forward	ACAGAGGGGAGAAGATGTGC	20
	Reverse	CCCTGGCAGACAGATTGG	
Flt3l	Forward	CCTAGGATGCGAGCCTTGT	102
	Reverse	TGTTTTGGTTCCCAACTCG	
Kitl	Forward	TCAACATTAGGTCCCGAGAAA	71
	Reverse	ACTGCTACTGCTGTCATTCCTAAG	
Cxcl12	Forward	GGTTCTTCGAGAGCCACATC	21
	Reverse	TGTTCTTCAGCCGTGCCAA	
Ccl25	Forward	GAGTGCCACCCTAGGTCATC	9
	Reverse	CCAGCTGGTGCTTACTCTGA	
Fgfr2IIIb	Forward	CCTGCGGAGACAGGTAACA	17
	Reverse	CGGGGTGTTGGAGTTCAT	
Wnt4	Forward	TCATGAATCTTCACAACAACGA	4
	Reverse	CCCCGTGACACTTGCACT	
Psmb11	Forward	CAGGGTTAGTTCTGGGAGCA	21
	Reverse	CAAAGCAGGAAACCCAAAAG	
Ctsl	Forward	CAAATAAGAATAAATATTGGCTTGTCA	60
	Reverse	TGTAGCCTTCCATACCCCATT	
MhcIIEb	Forward	CCTCCAGTGGCTTTGGTC	109
	Reverse	CCGTTGTAGAAATGACACTCAGA	
Aire	Forward	GGTTCCTCCCCTTCCATC	45
	Reverse	GGCACACTCATCCTCGTTCT	
Hmbs	Forward	TCCCTGAAGGATGTGCCTAC	79
	Reverse	AAGGGTTTTCCCGTTTGC	
Hprt	Forward	TCCTCCTCAGACCGCTTTT	95
	Reverse	CCTGGTTCATCATCGCTAATC	
Tbp	Forward	GGGGAGCTGTGATGTGAAGT	97
	Reverse	CCTGGTTCATCATCGCTAATC	

Table 2.23. Oligonucleotide sequences used to perform RT-qPCR on selected genes.

2.10.C.TCRβ V-region sequencing primers

2.10.C.I. Primer sequences for TCR amplicon generation

Primer	**Application**	**Sequences**
SMART-NNN	Template switch	AAGCAGUGGTAUCAACGCAGAGUNNNNUNNNNUNNNNUCTTrG$_{(3)}$
P7-SMART-Index	Forward	CAAGCAGAAGACGGCATACGAGATXXXXXXGGCGAAGCAGTGGTATCAACGCAGAGT
P5-mTCRBrev3	Reverse	AATGATACGGCGACCACCGAGATCTACACCTTGGGTGGAGTCACATTTCT

Table 2.24. Primer sequences used to generate cDNA libraries of TCR Vβ gene regions.

2.10.C.II. Index Sequences (Unique Molecular IDs: UMIs)

Index Number	Index sequence
S62	GTACGT
S63	TGAGCG
S64	TCAGAT
S65	ATAGAT
S66	TCTAGC
S67	CCTGAT
S68	GCTCTA
S69	ACTTCG
S70	TGTCAG
S71	CGTTGA
S72	GGTACT
S73	AGTGTC
S74	GATTAC
S75	TTCGAC
S76	CTCAGT
S77	GTCTCA
S78	ATCCTG
S79	TCCCCT
S80	GCCAAG
S81	ACCGGA
S82	TGCATA
S83	TACTGG
S84	CACCAA
S85	GACGTT
S86	AACACC
S87	TTGACG
S88	CTGGTA
S89	GTGCTA
S90	ATGTGC
S91	GCGGCC
S92	ACGATT
S93	TGGGGT
S94	CGGAAC
S95	AGGCCA

Table 2.25. Unique sequences of P7-SMART-Index primer. A different index number was used per sample processed to allow for multiplexing of samples during sequencing.

2.10.C.III. Custom primers used for MiSeq 2 x 300 bp sequencing

Primer	Application	Sequence
R1-mTCRB	Read 1	CGAGATCTACACCTTGGGTGGAGTCACATTTCT
UMI-read2	Read 2 (UMI only)	GGCGAAGCAGTGGTATCAACGCAGAGT
Index-smartrev	Index	ACTCTGCGTTGATACCACTGCTTCGCC

Table 2.26. Custom primers used during MiSeq sequencing of TCR amplicons.