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Analysis of Thymic Epithelial Cell Development Using a Transgenic Mouse Model With Enhanced Green Fluorescent Protein
이학석사학위 청구논문

녹색형광단백질발현 유전자형질전환 생쥐를 이용한
흉선상피세포 발생 연구

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Transgenic Mouse Model With Enhanced Green Fluorescent 
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Abstract

Thymus provides a specialized microenvironment for the development of T cell precursors. This developmental program depends upon interactions with thymocytes and stromal cells, most importantly thymic epithelial cells, which provide molecular signals for proliferation, survival and differentiation.

Here, 3.1Kb TSCOT EGFP transgenic mouse were established using Thymic Stromal Co-transporter (TSCOT) promoter in order to direct expression of EGFP in to the subpopulation of thymic epithelial cells.(4)

Using thymic epithelial cells specific marked transgenic mouse, I focused on where/when TSCOT expressing cells play roles during thymic compartmentalization. Subpopulation of thymic epithelial cells were analyzed for the EGFP expression in the different compartments of thymic microenvironment by flow cytometry and histology.

To identification of TECs progenitor or stem cells in 3.1kb TSCOT EGFP transgenic mouse, stem cell analysis were performed using Fetal Thymic Organ Culture (FTOC) and Side Population (SP). This transgenic mouse provides the unique system that can be used for isolating thymic epithelial cells and for the studies of thymic organogenesis and functional compartmentalization.
Introduction

The thymus is the major site for the development and production of T cells. This organ is composed of a complex framework of stromal cells that is filled with a transient population of differentiating thymocytes. Stromal cells are comprised of a number of different cell types and different regions of the thymus are composed of distinct sets of stromal cells. T-cell development is characterized by the progression through several phenotypically distinct stages, defined as double negative (DN), double positive (DP) and single positive (SP) based on expression of the co-receptors CD4 and CD8. The DN subset is further subdivided into four stages (DN1-3 and DN4/pre-DP) by differential expression of CD44 and CD25. (1, 6) Thymocytes at different stages of development occupy distinct spatially restricted domains in the adult thymus, indicating that differentiation occurs concomitantly with a highly ordered migration; T-cell precursors enter the thymus at the cortico-medullary junction, then migrate progressively to the subcapsular zone of the outer cortex, back through the cortex and into the medulla, from where they egress to the periphery. Thymocytes and TECs are in close contact throughout this differentiation programme. (7) It is believed that this organization provides a number of different microenvironments that affect T cell development as these cells transit through the thymus. Although thymocytes development has been extensively studied, not much is known regarding how stromal cells affect this process.

In order to address this question, it was used a molecular approach to clone gene that are preferentially expressed in thymic stromal cells. We reported the identification and characterization of a novel gene product (originally called TSC-1C12) from a mouse thymic stromal cell cDNA library. The mRNA of this gene contains a single open reading frame of 1987
bp that encodes a type III TM protein of 479 amino acids called Thymic Stromal Co-Transporter (TSCOT). (5, 9)) And With this TSCOT gene we generated 3.1Kb TSCOT promoter driven enhanced green fluorescent protein (EGFP) reporter system. Our question is where TSCOT expressing cell play roles during thymic compartmentalization. It was reported there is no TSCOT gene expression in CD45- but MHC II and in MHC II+ thymic epithelial cells and FoxN1 that well known as a nude gene express TSCOT gene in RT PCR analysis. And also in situ hybridization data shows endogenous TSCOT expression is in cortex in adult stage. In this background information, I focused on research about where/when TSCOT expressing cells play roles during thymic compartmentalization. Stromal cells consisting of various cell types are also present in the thymus. Thymic epithelial cells (TECs) are present in both cortical and medullary thymic areas, and are known as cortical TECs (cTECs) and medullary TECs (mTECs), respectively. These cells are likely to represent heterogeneous populations, as indicated by both antibody staining and ultrastructural morphology. I characterized TECs by flow cytometry and histology and subpopulation of thymic epithelial cells were analyzed for the EGFP expression in the different compartments of thymic microenvironment. Flow cytometry technique allows simultaneous multiparametric analysis of the single cells flowing through an optical and electronic detection apparatus and able to analysis several thousand particles every second, in real time, and can actively separate isolate particles having specified properties. A flow cytometry is similar to a microscope, except that instead of producing an image of the cell, flow cytometry offers high-throughput (for a large number of cells) automated quantification of set parameters.

Several molecules have been identified that are important in early thymus development, including forkhead N1 (FOXN1), T-box 1 (TBX1) and pre-B-cell leukaemia transcription factor 1 (PBX1). The thymus initially develops around embryonic day 10 (E10) to E11 of gestation as an
endodermal epithelial–cell bud that in encapsulated by mesenchyme. During embryonic development the thymus increases in size and compartmentalizes into cortical areas that are separated from inner medullary area by the cortico–medullary junction. The identification and isolation of TECs progenitors is not only important in understanding the mechanism regulating thymus development, but also for therapeutic strategies aimed at regenerating thymic tissue to boost new–T–cell depletion that is caused by ablative therapies or viral infection. (12) There are two basic models. One of them is endodermal progenitor cell might give rise to a committed common, thymic epithelial progenitor cell, which gives rise to all cortical and medullary TECs types either directly or through intermediate cortical and medullary epithelial progenitor cells. and the other models is endodermal progenitor might give rise directly to separate cortical and medullary epithelial progenitor cells. In either case, the progenitor population(s) might also contain stem cells, the potency of which is likely to be restricted to thymic epithelial, or possibly endodermal, lineages. (2, 13) For early stage of thymus for stem cell analysis, Fetal Thymic Organ Culture (FTOC) and Side Population (SP) techniques was used, FTOC has widely used to investigate the impact of T cell and TECs developments. (11) And Side population analysis, as stem cells are very important to the organism it is not surprising that they are thought to have very efficient membrane pumps to enable them to protect themselves from influx of potentially harmful chemicals. (10) Certain cell types are known to have highly efficient pumps for the dyes Rhodamine 123 and Hoechst 33342 in particular. Hoechst 33342 is a DNA–binding dye that binds preferentially to A–T rich regions of DNA. The dye is excited by UV wavelengths (maximum excitation is 395nm) and emits in the blue (emission maximum is 450nm). However, the emission wavelength shifts to the red end of the spectrum in certain conditions such as apoptosis and also when the dye concentration is high. This property is also exploited in the identification of putative stem cells in a variety of
human and murine cell types. Although Hoechst is able to enter live cells, it is also actively pumped out by ABC (ATP-Binding Cassette) transporters which include p-glycoprotein and ABCG2 in human cells. These transporters may also be specifically inhibited by agents such as verapamil and reserpine. Margaret Goodell’s group was the first to identify a population of cells in bone marrow that appears particularly effective at pumping out the Hoechst dye. These ‘side population’ or SP cells have been shown to possess stem cell characteristics (a lineage negative phenotype, enriched long-term culture initiating cells). Using these techniques, I applied the two basic theory into 3.1Kb TSCOT EGFP transgenic mouse model to check whether EGFP expressing cells have stem cell activity or not.
Materials and methods

Thymic stromal cell isolation
Thymuses were chopped with straight scissors and finely minced in cold PBS and washed several times, and thymocytes were removed by allowing the suspension to stand for 1 min. (If sample is turbid, that means that thymocytes need to be removed more). The remaining fragments were subjected to digestion with 0.25% trypsin (Sigma, St Louis, MO, USA) and 10 mg/ml deoxyribonuclease I (Sigma) for 20 min at 37°C. After adding 10% Fetal Bovine Serum (FBS), the resulting suspension was filtered and then centrifuged at 6500 r.p.m. for 20 s. and wash with 900㎕ PBS (10% FBS) and 100㎕ DNase then centrifuged at 6500 r.p.m for 20s. The pellet was incubated with 5㎕ of anti-Fe mAb 2.4G2 (X100) and 10㎕ of anti-mouse CD45 microbeads per 5e07 cells (Milteny Biotec, Sunnyvale, CA, USA) for 20 min at 4°C and CD45 cells were collected using magnetic bead cell sorting (MACS, MiltenyBiotec). Wash column with 2ml of FACS buffer. Collect total effluent in 2ml tubes. and centrifuged at 1500 r.p.m for 5 min and removed supernatant. Suspend with 200㎕ of FACS buffer and count (around 1:10 or 1:20 dilution) the cells and stain for FACS analysis. (3)

Flow cytometric analysis
Cells were washed in cold FACS buffer (PBS + 1% BSA) and centrifuged at 1200 r.p.m for 2 min, subsequently stained on ice with 5㎕ of 2.4G2 antibody (X1), stained with primary antibody for 20 min and wash with 200 μl of FACS buffer then centrifuged at 1200 r.p.m for 2 min, removed the supernatant and then stained with secondary antibody for 20 min, wash with 200μl of FACS buffer then centrifuged at 1200 r.p.m for 2 min. Pellets was moved to 5ml FACS tube and suspend with 400μl of FACS buffer and
analyzed on a FACS Aria (BD Biosciences, San Jose’, CA, USA) with one laser and UV in the presence of 1–2 mg/ml of propidium iodide (PI). Analyses were done using FlowJo 6.0 (http://flowjo.com).

**mAb**

The following mAbs were used: anti-CD45 (30–F11, rat IgG2b) PE–Texas Red conjugate, anti-CD45 (30–F11.1, rat IgG2b) conjugated with FITC. The anti-CD45 antibody was purchased from Caltag (Burlingame, CA, USA) and all others from BD PharMingen (San Diego, CA, USA). Anti-mouse CD45 microbeads was purchased from Milteny Biotec, Sunnyvale, CA, USA. Anti-aminopeptidase A (CDR1, IgG2a) were purchased from American Type Culture Collection (Rockville, MD, USA) and were prepared by Larry Lantz (Custom Antibody Services Facility, NIAID, NIH). Biotinylated UEA–1 was purchased from Vector Laboratories Burlingame, CT, USA). CLVE1 anti-TSCOT mAb (prepared by Dr. L lanz, NIAID), and PE-conjugated goat anti-Rat IgM (Jackson Laboratories).

**Mouse Fetal Thymic Organ Culture (FTOC)**

Harvest the thymus from 14.5 days embryos. Put embryo into 5ml PBS. And get rid of head with forcep then cut down the middle little bit and spread out the find thymus then take them out with forcep then put thymus in DMEM temporary. Prepare 5ml DMEM plus 1.35mM 2-deoxy guanosine. in the plate and sponges filters. And Put filters on the sponges (Shiny side should be on the top) and then put it in the plate. Then put lobes on the filters. (6 lobes/filter). Prepare plastic box and put the plate in plastic box. Then fill up with 10% Co2 in the box and taped around. Incubate 37 °C for 5 to 7 days.
Side Population (SP) Analysis

After 5-6 days culture treated with 2-deoxy guanosine, Collect thymus from the plates and thymic epithelial cells cell preparation. Prepare 10e6 cells/tube and stained with 5㎍/ml of Hoeschst 33342 for exactly 90min at 37 ℃. Wash with cold HBSS and centrifuged at 1500 rpm for 5 min. Then removed the supernatant and resuspend with 400㎕ of HBSS and FACS Analysis.

Generation of 3.1Kb TSCOT EGFP transgenic mice

The EGFP reporter construct (p1C12RIPST3.1EGFP) driven by the mouse Tscot promoter containing the DNA from the EcoRI site (32950) to the PstI site (+126) was prepared in two steps in order to avoid deletion of the internal PstI sites. The expression cassette containing the 3 kb Tscot promoter, EGFP, and the plasmid encoded polyA site was then isolated by cutting with EcoR1 and A£II. This fragment was used to produce transgenic mice at the NIAID Transgenic facility using C57BL/6 zygotes. (Chuan Chen, etc, 2000). Mouse was bred in an center for animal resource development, college of medicine, Seoul National University and in an AAALAC-approved animal reseach center, Sungkyunkwan University. Also, about 800 mouse are maintained in LMCI mouse facility in Inha University.
Results

Mouse model system

In this experiment was used 3.1Kb TSCOT EGFP transgenic mouse in this study. (4) EGFP expression was checked in several organs, EGFP expression is detected strongly in thymus. There were no EGFP expression in Spleen, kidney. (Fig. 1) Also, it was shown by Chan Sik Park that what kind of cells express EGFP, thymus was stained with anti CD45 Red to identify thymocyte and see with confocal microscopy, only thymic epithelial cells expressing EGFP. There is no EGFP expression in thymocyte.

(Chan Sik park, Department of pathology, (Asan Medical Center) college of medicine, university of ulsan, Unpublished)

Postnatal Development: Expression is compartmentalized and the expression patterns was changed by developmental stages

It was shown by Chan Sik Park that compartmental EGFP expressing in postnatal stages is changing. In 2 days old mouse, EGFP expression is wide spread in cortex and medullar areas, but 7 days when cortex is more mature, EGFP expression in cortex is decreased whereas EGFP expression in medullar is increased. This data indicated that EGFP expression is compartmentalized and the expression patterns was changed by developmental stages. Interestingly, endogenous TSCOT gene expression is in only cortex in adult mouse but in this transgenic mouse, it has TECs specific expression but expression patterns is totally different compare with endogenous TSCOT gene expression. (Chan Sik park, Department of
pathology, (Asan Medical Center) college of medicine, university of ulsan, Unpublished)

**Change in the EGFP levels during the postnatal development**

Based on past experiments data, it was difficult to see the cortex expression using histology method. So, Flow cytometry was used for single cell analysis with compartment specific marker for comparing with histology data. To study how each TECs establishes a different compartment at the single-cell level, I first assigned adult thymic stromal cells to either a cTECs or an mTECs phenotype using cell surface markers in a flow cytometry analysis. The reagents used for specific compartments were the CDR1 mAb for cTECs and the UEA-1 lectin, which is reported to bind to a sub-population of mTECs. And thymocyte is gated out by staining with CD45 and gated each cTECs, mTECs and nonTECs in stromal cells population.

The results showed that percentage of cTECs sub-population is NB 13.4%, 2W 38.5%, 4W 74.7% that is getting increased whereas percentage of mTECs and nonTECs is getting decreased in total stromal cells. And in EGFP+ histogram profiles, percentage of EGFP expressing cells, NB 35.6%, 2W 15.5%, 4W 11.8% was decreased in all developmental stages in histogram. It is accord with histology data that EGFP expressing cells are getting decreased as it aged. To see the changes of EGFP expression levels in each compartments, only EGFP positive population was gated and see with CDR1 and UEA-1. There was only background level of EGFP expression in nonTECs area but EGFP expression in cTECs was very high level whereas mTECs was low in newborn stage. However, mouse is getting older, cTECs population, NB 41.7%, 2W 10.1%, 4W 9.24% was getting decreased EGFP expressing cells whereas mTECs population, NB 15.2%, 2W 73.4%, 4W 78.8% was getting increased EGFP expressing cells.
This data also agreed with histology data that EGFP expressing cells in cTECs is getting decreased whereas mTECs is getting decreased as it aged. (Fig. 2).

**Quantitative analysis of cTECs & mTECs for EGFP expression in 3.1kb TSCOT EGFP thymic stroma**

To see the how many cells are expressing EGFP, quantitative analysis is used in each compartments. (Fig. 3) EGFP expression level is newborn 59%, 2W 21.8%, 4W 16.1% in cTECs. This data indicated EGFP expressing cells in cTECs was decreased as it aged. However, in mTECs expressing level is newborn 24.3%, 2W 31.4%, 4W 13.2%, temporally increased at 2w but decreased again at 4w. But the absolute number of EGFP expressing cells in mTECs is increased somehow. It is unknown why cTECs EGFP expression level is getting decreased while mTECs EGFP expression level is getting increased. Further studies are required to figure out the mechanism.

**Fetal thymic epithelial cells development**

To identification of TECs progenitor or stem cells in 3.1kb TSCOT EGFP transgenic mouse, stem cell analysis was used with early stage thymus to see whether EGFP expressing cells have stem cell activity or not. Fetal Thymic Organ Culture (FTOC) and Side Population (SP) analysis is used for this experiment. (11, 12) We prepared embryonic days 14.5 fetal thymus and treated 2-Deoxy guanosine to get rid of thymocyte. After 6 days culture, thymic epithelial cell preparation and staining with Hoechst 33342. (Fig. 4) C57BL/6 mouse was used as a control. As a results, Side
population is present both 3.1Kb TSCOT EGFP and C57BL/6 control mouse. (Fig. 5A) There is enough EGFP expression in 3.1Kb TSCOT EGFP mouse. However when only side population area were gated, there was no EGFP expression in side population area. Therefore, the conclusion is that at least there is no EGFP expression in side population in 3.1Kb TSCOT EGFP mouse. (Fig. 5B)

Co-expression of lineage specific markers in TSCOT+ cells suggests transitional cells

Related stem cell activity experiment, I have question where TSCOT expressing cells are located and what kind of markers TSCOT expressing cells have. It is reported by Moon Gyo Kim, when only TSCOT expressing population was gated and see with CDRI and UEA-1, TSCOT expressing cells are co-expressed with CDRI and UEA-1. (8) Most of the EGFP expressing cells are expressed in cortex but there is a population placed in both cortical and medullar areas. This population may transitional precursor cells and that population is getting decreased as it aged.
Discussion

In this experiment, thymic epithelial cells specific marked transgenic mouse model was established using Thymic Stromal Co-transporter (TSCOT) promoter and enhanced green fluorescent protein. Using this transgenic mouse I want to see when/where TSCOT expressing cell paly roles during thymic compartmentalization. During the Thymic epithelial cells development in 3.1Kb TSCOT EGFP transgenic mouse, EGFP expressing cells distribution was changed by developmental stages. While endogenous TSCOT expression is in cortex in adult stage. In 3.1Kb TSCOT EGFP transgenic mouse, EGFP expression was in wide spread in newborn stage. However, as time goes, after 7 days when cortex is more mature, cortical EGFP expression cells were decreased whereas medullar EGFP expression cells were getting increased in the histology analysis. This is very interesting fact that different expression patterns between endogenous TSCOT gene and 3.1Kb TSCOT EGFP transgenic mouse. Further studies are required to figure out the mechanism, such as cell cycle analysis and cell death analysis. Also using single cell analysis by FACS data support histology data.

There was no EGFP expressing cells in side population in 3.1Kb TSCOT EGFP transgenic mouse. That means that at least in 3.1Kb TSCOT EGFP transgenic mouse, there was no EGFP expression. but past data showed that in TSCOT positive gated profile, there was a population expressing both cortical marker CDR1 and medullary marker UEA-1 that may consider as transitional cells. This transitional cell population might give rise to a committed common, thymic epithelial progenitor cell, which gives rise to all cortical and mTECs types either directly or through intermediate cortical and medullary epithelial progenitor cells. We think that eventhough our 3.1Kb
TSCOT EGFP transgenic mouse don’t have stem cell activity but may act as a common progenitor cells. (Fig. 6) and another key unanswered question is related to the ongoing persistence and location of TECs progenitors in the adults thymus. and We think that TSCOT may act as a TECs stem cell or TECs progenitor’s marker. The use of cell-surface markers specific for TECs progenitors is essential to facilitate the identification of TECs progenitors and their subsequent isolation from later-stage thymus tissue. Further studies are required to address the stem cell or progenitors activity of EGFP expressing TECs in 3.1kb TSCOT EGFP transgenic mouse line and the stages in TECs development at which it may occur. Finally, further analysis of the mechanism regulating the development and function of TECs should help us to gain a better understanding of the processes that influence thymic development and organogenesis.
Fig 1. (A) Construct of 3.1Kb TSCOT EGFP transgenic mouse. (B) Thymus specific expression of EGFP in 8w 3.1Kb TSCOT EGFP transgenic mouse.
### Fig 2.

Single cell analysis using flow cytometry, percentage of EGFP expressing cells is decreased in all stages. It is accord with histology data, and then when only EGFP positive population were gated and see with CDR1 and UEA-1, there were only background level of EGFP expression in NonTECs area but EGFP expression level in cTECs area is very high and mTECs area is low in newborn stage. However, getting older, EGFP expressing cells in cTECs population is getting decreased whereas EGFP expressing cells in mTECs population is getting increased.
Fig 3. Quantitative analysis of cTECs & mTECs for EGFP expression in 3.1Kb TSCOT EGFP thymic stroma. Cortical TEC’s EGFP expressing level is newborn 59%, 2w 21.8%, 4w 16.1%, we can make sure EGFP expressing cells in cortex is decreased as time goes. By the way in mTEC expressing level is temporally increased at 2w and decrease again at 4w. But the absolute number of EGFP expressing cells in medullar is increased.
Fig 4. Schematic diagram of Fetal Thymic Organ Culture (FTOC) for fetal thymic epithelial cell development using side population method. Using E14.5 fetal thymus, treated with 2-deoxyguanosine to get rid of thymocyte. After 6 days culture, only stromal cells are collected by thymic epithelial cell preparation, then stained with Hoechst 33342 for stem cell analysis.
Fig 5. (A) Side population both 3.1Kb TSCOT EGFP and C57BL/6 control mouse. (B) Total EGFP expression level and EGFP expression level in side population. There was enough EGFP expression in whole population. However, when only side population area were gated and see the EGFP expression, there were no EGFP expression in side population area.
Fig 6. Thymic epithelial cell development. An endodermal progenitor cell might give rise to a committed common, thymic epithelial progenitor cell, which gives rise to all cTECs and mTECs types either directly or through intermediate cortical and medullary epithelial progenitor cells. Diagram is taken from Blackburn and Manley (2004)
References


