

Expression Analyses Revealed Thymic Stromal Co-Transporter/Slc46A2 Is in Stem Cell Populations and Is a Putative Tumor Suppressor

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By combining conventional single cell analysis with flow cytometry and public database searches with bioinformatics tools, we extended the expression profiling of thymic stromal co-transporter (TSCOT), *Slc46A2/Ly110*, that was shown to be expressed in bipotent precursor and cortical thymic epithelial cells. Genome scale analysis verified *TSCOT* expression in thymic tissue- and cell type- specific fashion and is also expressed in some other epithelial tissues including skin and lung. Coexpression profiling with genes, *Foxn1* and *Hoxa3*, revealed the role of *TSCOT* during the organogenesis. *TSCOT* expression was detected in all thymic epithelial cells (TECs), but not in the CD31⁺ endothelial cell lineage in fetal thymus. In addition, ABC transporter-dependent side population and Sca-1⁺ fetal TEC populations both contain *TSCOT*-expressing cells, indicating TEC stem cells express *TSCOT*. *TSCOT* expression was identified as early as in differentiating embryonic stem cells. *TSCOT* expression is not under the control of *Foxn1* since *TSCOT* is present in the thymic rudiment of nude mice. By searching variations in the expression levels, *TSCOT* is positively associated with *Grhl3* and *Irf6*. Cytokines such as IL1b, IL22 and IL24 are the potential regulators of the *TSCOT* expression. Surprisingly, we found *TSCOT* expression in the lung is diminished in lung cancers, suggesting *TSCOT* may be involved in the suppression of lung tumor development. Based on these results, a model for TEC differentiation from the stem cells was proposed in context of multiple epithelial organ formation.

INTRODUCTION

Thymus produces educated T cells that can react with peptide antigen loaded on a self major histocompatibility complex (MHC) but not with self antigens. Developing thymocytes are guided and selected in the microenvironment of thymic stromal cells. Among the stromal cells, thymic epithelial cells (TECs) are major components that plays important roles of thymocyte differentiation in the separate compartments, the cortex and the medulla.

TECs also play critical roles during thymic organogenesis as shown in *Foxn1* mutant mice, in which early TEC differentiation is abrogated, functional thymus lacks and, therefore, no T cell is present (Nehls et al., 1996). In mice, initial thymic structure begins to form with the TEC precursor cells originated from the third pharyngeal pouch around fetal day 10.5 (Blackburn and Manley, 2004; Gill et al., 2003; Rodewald, 2008; Su et al., 2001). At this stage, fetal thymus does not show clear medullary compartmentalization yet although the cells of medullary thymic epithelial cells (mTECs) in nature are found (Roberts et al., 2012). Fetal thymus begins to express the cortex-specific markers such as CDR1 in addition to general epithelial markers, EpCAM and MHCII (Ahn et al., 2008; Boehm, 2008; Lee et al., 2012; Yang et al., 2005). Later, thymus undergoes atrophy by aging after puberty and/or by damaging insults such as radiation or stress hormones (Blackburn et al., 2002; Cheng et al., 2010; Gill et al., 2003). However, thymus can also be rejuvenated by removing steroid sex hormones or removing organs that produce sex hormones (Berzins et al., 2002; Lynch et al., 2009; Sutherland et al., 2005). The functional thymic epithelial stem cell (sTEC) in the adult or aged animals were identified (Blackburn et al., 2002; Rodewald et al., 2001; Swann and Boehm, 2007; Ucar et al., 2014; Wong et al., 2014). It is important to identify the molecular marker present in the sTEC to understand the mechanism of thymic regeneration and to translate into the clinic for the recovery of important cellular immunity.

There has been much evidence that cortical TEC (cTEC) and mTEC are derived from the single precursor TECs (pTEC) or sTEC (Bleul et al., 2006; Rossi et al., 2006). While pTEC can be bipotent or specific lineage- committed (Park et al., 2013; Ucar et al., 2014), TEC development may be more progressive without instant commitment to a specific lineage (Alves et al., 2014). The original specific antibodies used for the identification of sTECs are MTS24 and MTS20 (Bennett et al., 2002; Gill et al., 2002). These TEC stem cells were located in the small

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medullary islets of very young thymus or corticomedullary junction of the adult thymus (Rodewald et al., 2001). The cytokeratin K5 and K8 are also important molecules for the identification of sTECs (Klug et al., 1998; 2002). It was also proposed that TEC stem cells reside in the MTS10⁺ cells in the medullary area.

It has been considered that *Foxn1* might be the master key transcription factor controlling TEC differentiation (Chen et al., 2009; Cheng et al., 2010; Corbeaux et al., 2010; Manley and Condie, 2010; Nowell et al., 2011; Ucar et al., 2014). Some other transcription factors such as *Pax1/9* and *Hoxa3*, and signaling molecules such as *Shh*, *Wnt*, *Bmp* and *Fgf* were also identified from the studies using the mouse lines with gene ablation (Hollander et al., 2006; Manley and Condie, 2010). Those molecules function from the stage of third pharyngeal pouch to the initial state of thymus formation. *Foxn1* appears to be important for the survival and proliferation of committed TECs at the stage of thymic organ maintenance. *Wnt4* is responsible for the expression of *Foxn1* (Balciunaite et al., 2002).

However, the presence of sTEC without *Foxn1* expression was recently shown by Bruno Keywisky's group by using a new feature for stem cells to be able to form spheres in 3D culture (Ucar et al., 2014). Methods which can isolate stem cells based on their functionality will provide thrust for the studies on the initiation of thymic organogenesis at the molecular level and on the detailed processes on how it behaves. Our understanding of sTEC is still in a primitive state. One of the distinguished common features of stem cells, either from the specific organs or even from cancer cells, is called "side population" (SP) in flow cytometry (Golebiewska et al., 2011; Zhou et al., 2001). The cells in the side population emit both blue and red fluorescence from the DNA staining dye, Hoechst33342. This phenomenon is mediated by the ABC transporters and can be blocked by the inhibitor (Golebiewska et al., 2011; Zhou et al., 2001). Therefore, it is very useful to identify stem cells when no well-characterized stem cell marker is available.

TSCOT (*Slc46A2/Ly110*) is a gene encoding cTEC-specific membrane protein (Ahn et al., 2008; Chen et al., 2000; Kim et al., 2000; Yang et al., 2005), isolated from the cDNA library of SCID thymus and of fetal thymic stroma (Kim et al., 1998; Park, 1997). Its expression peaks at the early stage of thymic development and reduces when the thymus is more mature (Ahn et al., 2008; Kim et al., 2000; Lee et al., 2012; Yang et al., 2005). When *LacZ* reporter is inserted in the *TSCOT* locus, β -galactosidase expression was found in the whole thymus of new born but only in the cortex and corticomedullary junction of adults (Ahn et al., 2008). When hooked to the promoter fragments (9.1 kb), evolutionarily conserved sequences located in the upstream of the coding sequence, reporter EGFP expression copied the expression pattern of endogenous gene while a shorter promoter fragment (3.1 Kb) revealed unexpected expression in the medulla at the adult stage of transgenic mice (Chen et al., 2000; Lee et al., 2012). The Cre recombinase under the control of *TSCOT* promoters resulted in expression of EGFP and β -galactosidase in the bipotent pTEC by the deletion of *loxP* sequences harbored in the *ROSA* locus of the transgenic mouse lines (Park et al., 2013). Therefore, the unique restricted pattern of the *TSCOT* expression is of high value to study TEC differentiation and thymic organogenesis.

Expression of *TSCOT* has also been noticed in the male epididymal duct in conventional Northern blotting and immunohistochemistry (Obermann et al., 2003), and the *TSCOT* locus has been assigned in a susceptibility of cervical carcinoma by human genetic analyses (Engelmark et al., 2006; 2008). In the

current era of bioinformatics, there has been many systemic data accumulating in the public database and available for analysis.

In this study, we took advantage of public database and bioinformatics tools and performed genetic profiling in addition to classical methodologies. We show *TSCOT* is expressed prior to bipotent pTEC, at the side population stage of thymic epithelial cells, and also even in differentiating ES cells. Its expression does not depend of *Foxn1*. *TSCOT* expression and its roles in other epithelial tissues like skin and lung are discussed.

MATERIALS AND METHODS

Expression profiling using public database

The data sets were obtained from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>), and the extension changed as txt files to analyze in the GENESIS program (version 1.7.6) released by Graz University of Technology Institute for Genomics and Bioinformatics. Genes and the probes used are shown in Table 1. Multiple sample data are averaged before the final analysis. The normalized data of the genes were sorted by similarity to *TSCOT* genes or calculated by using Hierarchical Clustering to generate heatmaps. Some of the GEO data sets are drawn as graphs and calculated P values with a two-tailed-T test in GraphPad Prism (version 6.0c).

Mice

The mouse lines TDLacZ (Ahn et al., 2008), 3.1T-EGFP (Chen et al., 2000), and 9.1T-NE (Lee et al., 2012) were maintained in the Laboratory of Molecular and Cellular Immunology Animal Facility of Inha University, Korea. All animal studies are in compliance with the Use of Laboratory Animals under the proper protocols. The protocols were approved by the Committees on the Ethics of Animal Experiments of NIH (LCMI Protocol 8) and Inha University (Protocol LMCI-2). Fetal mice were obtained from timed mating. The presence of a vaginal plug was considered at E0.5.

For genotyping, tail samples were extracted and used for a polymerase chain reaction with primers for the TDLacZ locus: Neo primer (ACCGCTATCAGGACATAGCGTTGG), 1C12 F1 (TACTCAAAGTGATGCTGGACTGG), 1C12 B2 (CCGAGGGTTCCTGGTACATTC), and the EGFP locus: EGFP-F (GCCACAAGTTCAGCGTGTCC), EGFP-R (GCTTCTGTTGGGTCTTTC), using the red Extract-N-Amp Tissue PCR kit (Sigma).

Automatic cell counting

A fluorescence-based, automatic cell counter (Luna-FL, Logos Biosystems) was used to measure accurately the numbers of cells including thymic epithelial cells. The contamination from red blood cells could be automatically excluded because this system enumerates only nucleated cells.

Thymic stromal cell preparation

A single cell suspension was prepared as described (Lee et al., 2012). Briefly, thymic tissues or deoxyguanosine treated fetal thymic organ culture were treated with 0.25% trypsin (Invitrogen) for about 20 min, in the presence of DNase I (Sigma), and washed with phosphate buffered saline (PBS) containing 10% fetal bovine serum (FBS). For further purification of TEC, the single cell suspension was isolated using magnetic bead cell sorting after incubating with anti-Fc mAb 2.4G2 and anti-mouse CD45 microbeads (Milteny Biotec) for 20 min at 4°C.

Table 1. List of Selected Gene Probe IDs used in the bioinformatics analyses

GPL*	Gene symbol	Probe ID	GenBank access number
GPL570	<i>AIRE</i>	208090_s_at	NM_000658
	<i>BMP4</i>	211518_s_at	D30751
	<i>CD248</i>	219025_at	NM_020404
	<i>CLDN18</i>	214135_at	BE551219
	<i>CLIC5</i>	219866_at	NM_016929
	<i>CRIP3</i>	235720_at	AI042209
	<i>CRTAC1</i>	221204_s_at	NM_018058
	<i>CYP4B1</i>	1555497_a_at	AY151049
	<i>EYA1</i>	214608_s_at	AJ000098
	<i>FGF7</i>	205782_at	NM_002009
	<i>FGF8</i>	208449_s_at	NM_006119
	<i>FOXG1</i>	206018_at	NM_005249
	<i>FOXN1</i>	207683_at	NM_003593
	<i>GKN2</i>	238222_at	AI821357
	<i>GRHL3</i>	232116_at	AL137763
	<i>HOXA3</i>	208604_s_at	NM_030661
	<i>HOXC13</i>	219832_s_at	NM_017410
	<i>HSD17B6</i>	205700_at	NM_003725
	<i>IL22</i>	221165_s_at	NM_020525
	<i>IL6</i>	205207_at	NM_000600
	<i>IL7</i>	206693_at	NM_000880
	<i>IRF6</i>	1552478_a_at	NM_006147
	<i>ISL1</i>	206104_at	NM_002202
	<i>LIF</i>	205266_at	NM_002309
	<i>LRRK2</i>	229584_at	AK026776
	<i>NOTCH3</i>	203238_s_at	NM_000435
	<i>OSM</i>	230170_at	AI079327
	<i>PAX1</i>	1553492_a_at	NM_006192
	<i>PAX9</i>	207059_at	NM_006194
	<i>PEBP4</i>	227848_at	AI218954
	<i>PLA2G1B</i>	206311_s_at	NM_000928
	<i>SFTPC</i>	215454_x_at	AI831055
	<i>SHH</i>	207586_at	NM_000193
	<i>SIX1</i>	205817_at	NM_005982
	<i>SLC46A2</i>	223816_at	AF242557
	<i>SOX2</i>	228038_at	AI669815
	<i>SUSD2</i>	234310_s_at	AK026431
	<i>TBX2</i>	207662_at	NM_005992
	<i>VEPH1</i>	232122_s_at	AK022666
	<i>WNT4</i>	208606_s_at	NM_030761
	<i>WNT5A</i>	205990_s_at	NM_003392
<i>WNT5B</i>	221029_s_at	NM_030775	
GPL1261	<i>Aire</i>	1419241_a_at	NM_009646
	<i>Bcl2</i>	1422938_at	NM_009741
	<i>Bmp4</i>	1422912_at	NM_007554
	<i>CD248</i>	1417439_at	NM_054042
	<i>Crip3</i>	1451410_a_at	AF367970
	<i>Dab2</i>	1420498_a_at	NM_023118
	<i>Dkk3</i>	1417312_at	AK004853
	<i>Eya1</i>	1421727_at	NM_010164
	<i>Fgf7</i>	1422243_at	NM_008008
	<i>Fgf8</i>	1451882_a_at	U18673
	<i>FoxG1</i>	1418357_at	NM_008241
	<i>FoxN1</i>	1450508_at	NM_008238
	<i>Gas1</i>	1416855_at	BB550400

(continued)

GPL*	Gene symbol	Probe ID	GenBank access number
GPL1261	<i>Hoxa3</i>	1452421_at	BB496114
	<i>HoxC13</i>	1425874_at	AF193796
	<i>IL6</i>	1450297_at	NM_031168
	<i>IL7</i>	1422080_at	NM_008371
	<i>Irf6</i>	1418301_at	NM_016851
	<i>Isl1</i>	1422720_at	BQ176915
	<i>Lif</i>	1450160_at	AF065917
	<i>Ly75</i>	1449328_at	NM_013825
	<i>Meis1</i>	1443260_at	BB055155
	<i>Notch3</i>	1421964_at	NM_008716
	<i>Osm</i>	1438767_at	BB237825
	<i>Pax1</i>	1449359_at	NM_008780
	<i>Pax9</i>	1421246_at	BC005794
	<i>Pbx1</i>	1449542_at	NM_008783
	<i>Psmb11</i>	1453150_at	BG069341
	<i>Shh</i>	1436869_at	AV304616
	<i>Six1</i>	1427277_at	BB137929
	<i>Six4</i>	1456862_at	AI893638
	<i>Slc46a2</i>	1423476_at	BB329435
	<i>Sox2</i>	1416967_at	U31967
	<i>Tbx1</i>	1425779_a_at	AF326960
	<i>Tert</i>	1450254_at	NM_009354
	<i>Wnt4</i>	1450782_at	NM_009523
<i>Wnt5a</i>	1436791_at	BB067079	
<i>Wnt5b</i>	1422602_a_at	NM_009525	
GPL2987	<i>FOXN1</i>	hCG31797.3	NM_003593.2
	<i>HOXA3</i>	hCG1640627.4	NM_153632.1, NM_030661.3, NM_153631.1
GPL8217	<i>PAX9</i>	hCG20991.2	NM_006194.1
	<i>SLC46A2</i>	hCG29190.4	NM_033051.2
GPL8217	<i>FOXN1</i>	HSG00201177 (ROSETTAGENE MODEL_ID)	NM_006015
	<i>HOXA3</i>	HSG00314123 (ROSETTAGENE MODEL_ID)	NM_002309
	<i>PAX9</i>	HSG00282340 (ROSETTAGENE MODEL_ID)	NM_030775
GPL8217	<i>SLC46A2</i>	HSG00262163 (ROSETTAGENE MODEL_ID)	NM_033051

*GPL, GEO platform accession number

Flow cytometry

Monoclonal antibodies used in the staining of cells include anti-MHCII (I-A^b), anti-CD45 (Ly-5), and anti-Sca-1. The antibodies were purchased from Caltag or from BD PharMingen. Anti-aminopeptidase A (CDR-1) and anti-EpCAM (G8.8) were prepared in the Custom Antibody Services Facility, NIAID, NIH. Biotinylated UEA-1 was purchased from Vector Laboratories.

Cells were washed in cold FACS buffer (PBS + 1% BSA), subsequently stained on ice with the primary and the secondary antibodies, then analyzed on FACSCalibur or FACSAriaII with two lasers in the presence of 1-2 µg/ml of propidium iodide

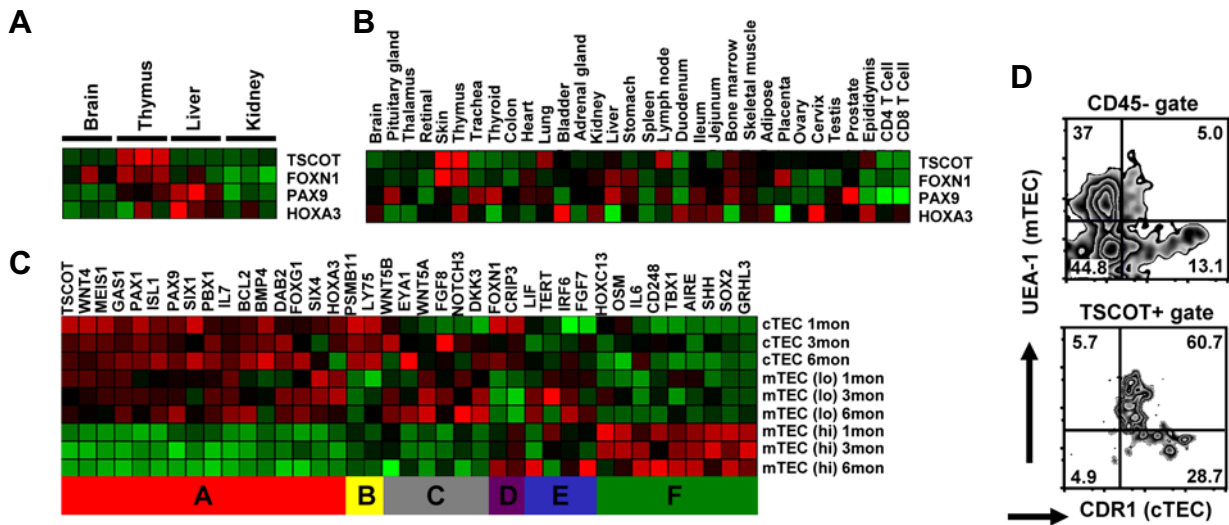


Fig. 1. Tissue and cell type specific TSCOT expression profiling. (A) Clustering of *TSCOT* with three other genes, *FOXN1*, *PAX9*, *HOXA3*, expression in human fetal tissues (GSE7905). (B) Expression in human adult tissues (GSE14938). (C) Gene expression during TEC development. cTEC and mTEC from adult mice are analyzed (GSE 56928). mTEC (lo): CD80⁺ and MHC-II⁺, mTEC (hi): CD80⁺ and MHC-II⁺. Gene expression profiles are from GEO microarray data. The gene expression values are normalized as 2.0 to -2.0 in the Genesis program. High, Red; Middle, Black; Low, Green. (D) Flow cytometric analysis of 4 weeks old thymic stromal cells for the lineage TEC makers. CDR1 is for cTEC, UEA-1 is for mTEC. The left panel shows CD45⁺ gate of whole stromal cells and the right panel shows the TSCOT⁺ gate.

(PI). Anti-Fc, 2.4G2 antibody was included in all flow cytometry staining to block Fc receptor. For side population analysis, a 1×10^6 dissociated single cell suspension of fetal thymic organ culture (FD14.5) were incubated for an hour at 4°C in the presence of 5 µg/ml Hoechst 33342 dissolved in Hanks balanced salt solution. For verification of the side population, verapamil 0.25 mM was included. After washing at 4°C, cells were resuspended and examined by a flow cytometer equipped with a UV laser (FACSArial). For multicolor staining with SP analysis, cells were pre-stained with selected antibodies including home-made mAb CLVE (Yang et al., 2005). Negative control of TSCOT staining was carried out with all the same combination of antibodies except mAb CLVE. Analyses were done using the FlowJo program (<http://flowjo.com>).

RT-PCR

Sorted 1000 cells were used for RNA preparation. cDNA was generated with Superscript III and RT-PCR was carried out with the primers for *TSCOT*: F84 (5-CAGTCTTCCAATAACCTGCTTTGGCCT-3) and B83 (5-CGATTCCATGTGCCCATTTG-3) to amplify a 310 bp fragment and for GAPDH (Ahn et al., 2008; Kim et al., 2000). The primers for *TSCOT* are located in the separate exons with one intron and RT⁻ control sampled did not show any band in the gel.

Histostaining and microscopy

The immunofluorescence and X-gal staining the sections is described (Lee et al., 2012). An isolated thymus was washed in PBS and fixed in 1% para-formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40, 1 mM MgCl₂ in PBS for 1 or 2 h on ice and was embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, USA). The 4 µm sections were fixed for 2 min in 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40 1 mM NaCl, then incubated with X-gal solution (1 part X-gal 40 µg/ml in dimethyl formamide, in 40 parts 2 mM MgCl₂, 5 mM potassium

ferricyanide, 5 mM potassium ferrocyanide in PBS) at 37°C for 48 h. For the detection of EGFP for fetal thymus sections, confocal microscopy was performed on the frozen sections in NIAID confocal facility (Leica SP2).

RESULTS

Gene profiling analysis verifies the tissue-specific TSCOT expression

In order to study the expression pattern of *TSCOT* at the genome level, we first used Google to identify any data and downloaded from the public database (<http://www.ncbi.nlm.nih.gov/geo/>) that shows the differential expression pattern (GSE7905). From the GEO database, the fetal tissue-specific expression was first examined (Fig. 1A). *TSCOT* expression was found only in the human fetal thymus, not in the fetal brain, fetal liver, nor fetal kidney. *FOXN1* expression showed a similar but not an identical pattern. *PAX9* and *HOXA3* that are previously associated with the third pharyngeal pouch formation are even more different from the *TSCOT* pattern. In human adult tissues (GSE14938), *TSCOT* was found in the thymus and skin. In addition, it was present in lung and epididymis at lower levels (Fig. 1B). *FOXN1* expression is also strongly expressed in the skin and thymus. However, it is not strongly expressed in any of the tissues that *TSCOT* is expressed at in the lower levels. Instead, *FOXN1* is expressed in the liver, stomach and placenta. These suggest that *TSCOT* and *FOXN1* may not be strongly associated in differentiated adult tissues.

Expression profiles of *TSCOT* and selected genes are investigated for the expression in the isolated mouse thymic epithelial cells (GSE56928). The genes for profiling (Table 2) are selected based on the literature which contains the information on the expression of the genes in the thymic epithelium or third pharyngeal pouch (references in Table 2). As shown in Fig. 1C, expression patterns are clustered as six different groups. A

Table 2. List of genes used in expression profiling during organogenesis

Gene* name	Full name	Function	Reference	TSCOT expression from GEO data
<i>AIRE</i>	Autoimmune regulator	Regulate mTEC development and differentiation, Transcription factor	Gordon and Manley, 2011; Sun et al., 2013	
<i>BCL2</i>	Growth Arrest-Specific 1	Antiapoptotic gene	Wong et al., 2014	
<i>BMP4</i>	Bone morphogenic protein 4	Essential for thymus and parathyroid morphogenesis prior to Foxn1	Gordon et al., 2010; Gordon and Manley, 2011	Higher TSCOT level in BMP4 treated 10T1/2 stem cells (GDS3025/ GSE5921) (P: 0.4685)
<i>CD248</i>	CD248 Molecule, Endosialin	Required for postnatal thymic growth and regeneration following infection-dependent thymic atrophy	Liu et al., 2014	
<i>CRIP3 (TLP)</i>	Cystein-Rich Protein 3 (Thymus Lim Protein)	Appears to have a role in normal thymus development	Kirchner et al., 2001	
<i>DAB2</i>	Mitogen-Responsive Phosphoprotein, Homolog	Wnt-inhibitors, Control proliferation and differentiation of stem cells into lineage-restricted cells	Wong et al., 2014	
<i>DKK3</i>	Dickkopf WNT Signaling Pathway Inhibitor 3	Wnt-inhibitors, Control proliferation and differentiation of stem cells into lineage-restricted cells	Wong et al., 2014	
<i>EYA1</i>	Eyes absent 1 homolog	Necessary for 3rd pouch development	Wei and Condie, 2011; Gordon and Manley, 2011	
<i>FGF7 (KGF)</i>	Keratinocyte growth factor	Induces mature and immature TECs and promotes differentiation of immature TECs	Rossi et al., 2006	
<i>FGF8</i>	Fibroblast growth factor 8	Indirectly influence TECs by regulating neural crest cells survival and differentiation, relate to early pouch formation	Gordon and Manley, 2011; Sun et al., 2013	
<i>FOXP1</i>	Forkhead Box G1	May play a role in the regulation of TEC differentiation during fetal and postnatal stages, Transcription factor	Wei and Condie, 2011	
<i>FOXP1</i>	Forkhead Box N1	Necessary for the development of immature TEC progenitor cells into cTECs and mTECs, Transcription factor	Blackburn et al., 1996; Bennett et al., 2002; Gordon and Manley, 2011; Bredenkamp et al., 2014	
<i>GAS1</i>	Growth Arrest-Specific 1	Cell-cycle suppressor gene	Wong et al., 2014	
<i>GRHL3 (Get-1)</i>	Grainyhead-Like 3	Ancient mediator of epithelial integrity, Transcription factor	Yu et al., 2008; de la Garza et al., 2012	Reduced TSCOT level in Get-1 KO skin (GDS2629/GSE7381) (P: 0.0042**)
<i>HOXA3</i>	Homeobox A3	Early pouch patterning and initial organ formation, Transcription factor	Manley and Capecchi, 1995; Su et al., 2001; Gordon and Manley, 2011	
<i>HOXC13</i>	Homeobox C13	Mediates transcriptional regulation of Foxn1, Transcription factor	Potter et al., 2010	
<i>IL22</i>	Interleukin 22	Leads to regeneration of supporting epithelial microenvironment for enhanced thymopoiesis after thymic injury	Dudakov et al., 2012	Reduced TSCOT level of IL22 treated epidermal keratinocytes (GDS2611/ GSE7216) (p < 0.0001****)
<i>IL6</i>	Interleukin 6	Associated with thymic involution	Chinn et al., 2012	
<i>IL7</i>	Interleukin 7	Cofactor for V(D)J rearrangement of the T cell receptor beta during early T cell development	Huang and Muegge, 2001; Zamisch et al., 2005	

(continued)

Table 2. List of genes used in expression profiling during organogenesis

Gene* name	Full name	Function	Reference	TSCOT expression from GEO data
<i>IRF6</i>	Interferon regulatory factor 6	Key determinant of keratinocyte proliferation-differentiation switch, Transcription factor	Richardson et al., 2006	Reduced TSCOT level in IRF6 KO skin (GDS2359/GSE5800) (P< 0.0001****)
<i>ISL1</i>	ISL LIM Homeobox 1	May play a role in the regulation of TEC differentiation during fetal and postnatal stages, Transcription factor	Wei and Condie, 2011	
<i>LIF</i>	Leukemia inhibitory factor	Maintenance mouse ES cell pluripotency, Associated with thymic involution	Shen and Leder, 1992; Graf et al., 2011; Chinn et al., 2012	Increased TSCOT level in murine CGR8 ES cells treated LIF (GDS3729/ GSE6689) (P: 0.1181)
<i>LY75</i> (<i>NLDC205</i> , <i>DEC205</i>)	Lymphocyte antigen 75	Contribute to antigen presentation, Marker of cTEC in adult thymus	Jiang et al., 1995; Shakib et al., 2009	
<i>MEIS1</i>	Myeloid ecotropic viral integration site 1	Functional and physical partners of Pbx1 and Hoxa3, Required for maintenance of the postnatal thymic microenvironment, Transcription factor	Hirayama et al., 2014	
<i>NOTCH3</i>	Notch homolog protein 3	Regulate murine T cell differentiation and leukemogenesis	Bellavia et al., 2008	
<i>OSM</i>	Oncostatin M	Plays an inhibitory role in normal and malignant mammary epithelial cell growth in vitro, Associated with thymic involution	Liu et al., 1998; Chinn et al., 2012	
<i>PAX1</i>	Paired Box 1	Early pouch formation and parathyroid development, minor role in thymus size, Transcription factor	Wallin et al., 1996; Gordon and Manley, 2011	
<i>PAX9</i>	Paired Box 9	Pouch and initial organ formation, TEC differentiation, Transcription factor	Hetzer-Egger et al., 2002; Gordon and Manley, 2011	
<i>PBX1</i>	Pre-B-cell leukemia homeobox	Required for embryonic thymic organogenesis, Transcription factor	Hirayama et al., 2014	
<i>PSMB11</i> (<i>β5t</i>)	Proteasome (prosome, macropain) subunit, beta type, 11	Positive selection of CD8+ T cells, cTEC specific proteosome subunit	Murata et al., 2007; Shakib et al., 2009	
<i>SHH</i>	Sonic hedgehog	Regulate pharyngeal region development	Moore-Scott and Manley, 2005; Gordon and Manley, 2011	Increased TSCOT level in SHH treated human fibroblasts (GDS4512/ GSE29316) (P: 0.1122)
<i>SIX1/4</i>	Sine oculis-related homeobox 1/4	Necessary for 3rd pouch development, Transcription factor	Wei and Condie, 2011; Gordon and Manley, 2011	
<i>SOX2</i>	SRY (sex determining region Y)-box 2	Regulate self-renewal of the mouse and human ESCs, important for the maintenance of stem cells in multiple adult tissue, establish induced pluripotent stem cells, Transcription factor	Cimpean et al., 2011; Liu et al., 2013	Higher TSCOT level in SOX2+ follicle dermal cells (GDS3753/ GSE18690) (P: 0.0015**)
<i>TBX1</i>	T-box transcription factor	Pouch formation and patterning, might establish parathyroid fate, Transcription factor	Jerome and Papaioannou, 2001; Hollander et al., 2006; Gordon and Manley, 2011	

(continued)

Table 2. List of genes used in expression profiling during organogenesis

Gene* name	Full name	Function	Reference	TSCOT expression from GEO data
<i>TERT</i>	Telomerase Reverse Transcriptase	Telomerase reverse transcriptase	Wong et al., 2014	
<i>WNT4</i>	Wingless-type MMTV integration site family, member 4	Controls thymopoiesis and thymus size by regulating TEC, thymocyte and their progenitor proliferation, regulate <i>Foxn1</i> expression in TECs	Sun et al., 2013	
<i>WNT5A</i>	Wingless-type MMTV integration site family, 5A	Regulate the survival of $\alpha\beta$ lineage thymocytes, regulator of cell growth in hematopoietic tissue	Liang et al., 2007	
<i>WNT5B</i>	Wingless-type MMTV integration site family, 5B	Produced by TECs and thymocytes, regulate <i>Foxn1</i> expression in TECs	Gordon and Manley, 2011; Sun et al., 2013	

*Gene names are listed in alphabetical order.

group contains *TSCOT*, *Wnt4*, *Meis1*, *Gas1*, *Pax1*, *Isl1*, *Pax9*, *Six1*, *Pbx1*, *IL7*, *Bcl2*, *Bmp4*, *Dab2*, *FoxG1*, *Six4* and *Hoxa3*. These genes are expressed in both cTEC and mTEC^{lo}. Among them, *TSCOT*, *Wnt4*, *Meis1*, and *Pax1* showed the strongest expression in the cTEC of the youngest mouse. Our earlier study on the expression kinetics (Kim et al., 2000) is consistent with these results. In this group, *Pax1*, *Pax 9*, *Six1*, *Meis1* and *Hoxa3* are the genes involved in the pouch stages (Manley et al., 2004). *Wnt4* and *Bmp4* were shown to be involved in the thymic organogenesis at the upstream of *Foxn1* (Bleul and Boehm, 2005). It is interesting to note that *Dab2* is a Wnt inhibitor. *Gas1*, *Bcl2* and *IL7* are the genes involved in the general cell cycle and survival. Group B contains *Psmb11* ($\beta 5t$) and *Ly75* (NLDC202/DEC205) that are genuine cTEC-specific genes. Group C (*Wnt5a*, *Wnt5b*, *Eya1*, *Fgf8*, *Notch3* and *Dkk3*) includes genes that are expressed higher in the later stages of cTEC and mTEC^{lo}. *Eya1* is known for roles in the third pharyngeal pouch (Gordon and Manley, 2011; Wei and Condie, 2011). However, its expression profile is somewhat different from the other genes involved in the same stage. Next, group D contains *Foxn1* and *Crip3* (*TLP*) that show the expression in cTEC and mTEC^{hi}. Here again, it clearly shows a deviation of expression pattern between *TSCOT* and *Foxn1*. Group E genes (*Lif*, *Tert*, and *Fgf7*) show the highest expression in the mTEC^{lo} or mTEC^{hi}. Given the known functions of *Tert* high expression in less divided cells, this result suggests that mTEC^{lo} may be found in more immature cells. The last group, group F contains *HoxC13*, *Osm*, *IL6*, *CD248* (*Endosialin*), *Tbx1*, *Aire*, *Shh*, *Sox2*, and *Ghrh3*. Those genes show the highest expression in the mTEC^{hi} population. *HoxC13* regulates *Foxn1* expression, and three genes, *Osm*, *IL6*, and *CD248* are involved in thymic atrophy and involution. The roles of *Tbx1* and *Shh* in mTEC^{hi} are not completely understood yet except that they are known for involvement in pouch formation.

The expression of *TSCOT* in mTEC^{lo} is not so surprising since it was found in the corticomedullary junction of young adult thymus where precursor or stem cells for thymic epithelium resides. When 4 week old thymic stromal cells were investigated by flow cytometry, the CD45⁺ population contains transitional cells with both cortical and medullary markers (CDR1⁺ UEA-1⁺). Those cells are included in the TSCOT⁺ gated cells beside CDR1⁺ UEA-1⁺ cTECs (Fig. 1D).

From these analyses, it was concluded that *TSCOT* is ex-

pressed in cTEC and undifferentiated and/or precursor mTEC. These expression profiles are common among the genes involved in early thymic organogenesis.

TSCOT is expressed in all TEC-committed stromal cells in fetal thymus

Next, we investigated the expression of *TSCOT* and reporters at the fetal stages in the different mouse models that we have previously characterized for the postnatal stages. The β -galactosidase reporter expression in TDLacZ thymus is restricted in the thymus as two dots at FD11 (Ahn et al., 2008). Figure 2A shows the β -galactosidase expression in the thymic sections at FD14.5. Expression of β -galactosidase is evenly distributed in the whole thymus, indicating most, if not all, the thymic epithelial cells at this stage express β -galactosidase. Another reporter mouse line, 3.1T-EGFP, which expresses EGFP in all TECs at the newborn stage (Park et al., 2013), showed EGFP expression earlier during fetal stages (Fig. 2B). At FD14 and 17, EGFP expression is also evenly distributed in the whole thymus. These results are consistent with the conclusion we previously described in which *TSCOT* is expressed in the pTEC stage (Park et al., 2013).

Fetal thymic stromal cells from normal C57BL/6 mouse (FD14) were analyzed for *TSCOT* expression with specific mAb CLVE (Yang et al., 2005). At this stage, EpCAM⁺ cells were all TSCOT⁺ (data not shown). When CD45⁺ stromal cells were displayed for CD31 as an endothelial lineage marker along with MHCII, it became clear that *TSCOT* expression is present in all MHCII⁺ cells and CD31⁺ MHCII⁺ cells (Fig. 2C). Only CD31⁺ MHCII⁺ cells of endothelial lineage were TSCOT⁺. From these results, it is concluded that endothelial cells either lost *TSCOT* expression due to lineage commitment from the common stem cell or originated from other type of precursor cells that do not express *TSCOT*.

TSCOT is expressed in the side population of TEC preparation

By using the TEC preparation from the deoxyguanosine treated FTOC of FD14.5, the presence of SP was tested with Hoechst 33342. In Fig. 3A, SP, which is ABC transporter sensitive, is clearly visible. When the inhibitor Verapamil was included, SP had decreased to 0.21% from 1.45%. Side population analyses were also applied with the TEC preparation using the same type culture of fetal thymus from 9.1T-NE mouse that shows

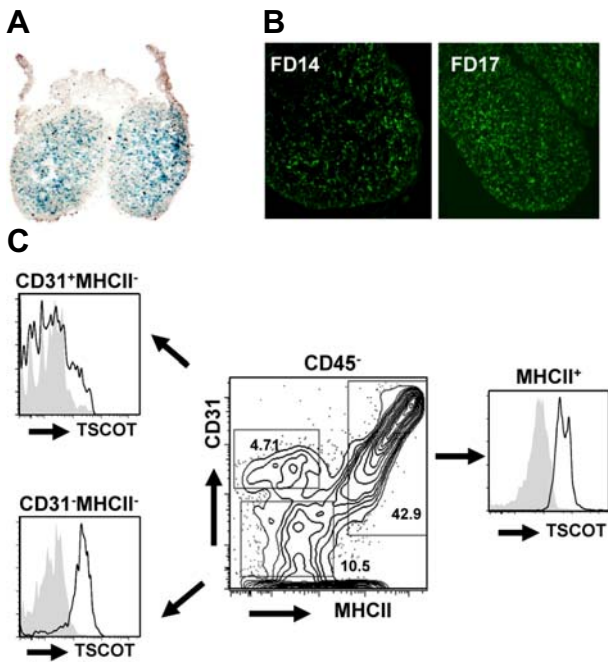


Fig. 2. TSCOT is expressed in fetal TEC committed cells. (A) LacZ expression in the FD14 thymus of TDLacZ mouse. (B) EGFP expression in the fetal 3.1T-EGFP transgenic thymus. (C) Flow cytometric analysis of fetal TEC preparation for TEC and endothelial lineages. CD31 is for endothelial cells, MHCII is for TEC cells. The histogram of negative population (gray area) is from the analysis of the same cell stained with the same sets of antibodies except mAb CLVE.

EGFP expression patterns in the same way as endogenous TSCOT (Lee et al., 2012). As shown in Fig. 3B, a portion of the SP of 9.1T-NE TECs expresses EGFP when compared with that of normal C57BL/6 TEC preparation. In contrast, the side population of 3.1T-EGFP TEC population did not show any EGFP expression (data not shown).

When SP analysis was carried out along with antibody staining, TSCOT expression in SP and the major population (MP) were also clear. SP, either MHCII negative or positive, showed specific TSCOT expression with mAb CLVE. In addition, 84% of the SP cells and 95% of the MP cells were TSCOT⁺ (Fig. 3C). The next experiment was to verify TSCOT expression at the RNA level using a sorted side population of TECs prepared from normal C57BL/6. RT-PCR using the RNA prepared from the sorted cells clearly showed TSCOT expression in SP and in MP (Fig. 3D).

Many different types of stem cells express Sca-1 marker and a recent report mentioned that a TEC progenitor population is Sca-1⁺ (Golebiewska et al., 2011). We also tested expression of Sca-1 in fetal TEC preparation (Fig. 3E). Sca-1⁺ populations are present in both EpCAM⁺MHCII⁺ and EpCAM⁺MHCII⁻ populations and most of them are TSCOT⁺. From these results, fetal TEC contains significant portion of sTECs that are TSCOT⁺.

TSCOT is expressed in differentiating embryonic stem cells

We searched the available data on TSCOT expression in the embryonic stem cell (ES) population. Two GEO sets of data (GSE14503 and GSE9440) contain an expression profile of T3 ES cell culture, embryonic body formation, and differentiating T3 ES cell into pancreatic islet-like cell clusters or fibroblasts (Fig. 4A). Expression of TSCOT is found in embryonic bodies but not in the undifferentiated ES nor in differentiated pancreatic islets and fibroblasts. TSCOT expression is clustered with

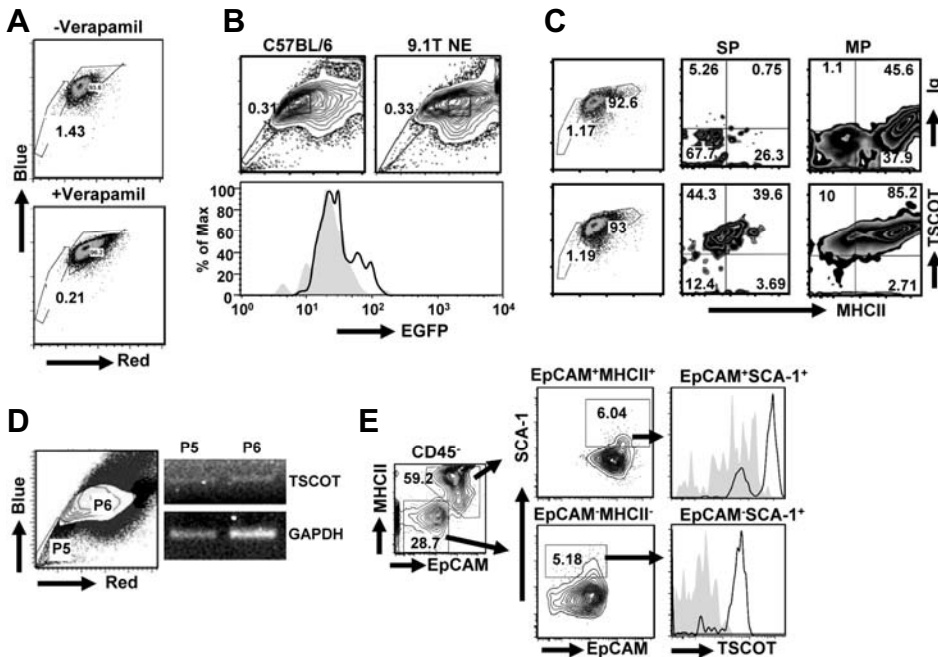


Fig. 3. SP analysis of TEC preparation. (A) SP analysis of fetal TEC preparation. Verapamil was included for blocking ABC transporter function during staining with Hoechst 33342. (B) EGFP expression of SP in the fetal TEC preparation from 9.1T-NE. EGFP levels are compared with the SP from C57BL/6. (C) A multicolor analysis of SP with pre-stained markers. SP and MP are shown. Top panels are stained samples without mAb CLVE. Bottom panels are with mAb CLVE. (D) RT-PCR analysis of sorted SP and MP from normal fetal TEC preparation. (E) Sca-1 population expresses TSCOT. Fetal TEC preparation gated for the CD45⁻ population and separated with EpCAM and MHCII (left). Each gate was analyzed for Sca-1 expression (middle) and for TSCOT (right). Grey histograms were obtained from the negative control sample stained without mAb CLVE.

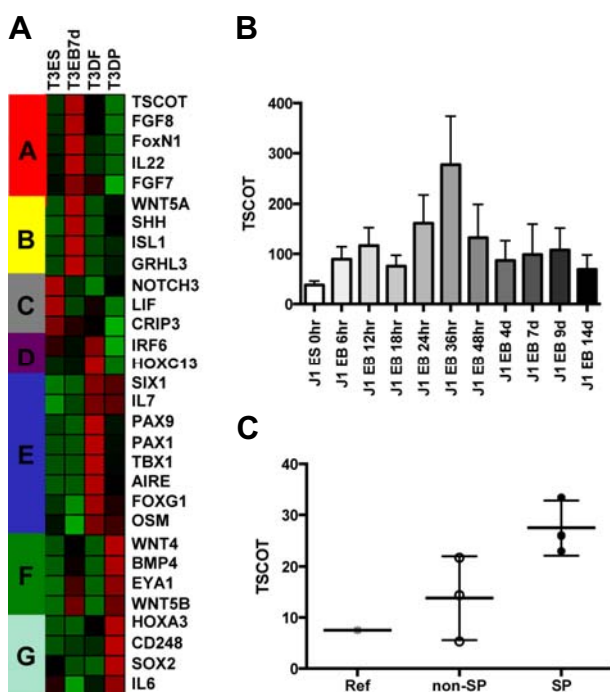


Fig. 4. Gene clustering analysis of ES and differentiating ES cells. (A) Gene expression level of pancreatic islet-like cell clusters and fibroblast-like cells derived from human T3 embryonic stem cells (GSE14503 and GSE9440). T3ES: human T3 embryonic stem cell, T3EB7d: day 7 embryonic body derived from T3ES, T3DP: Pancreatic islet-like cell clusters derived from T3ES, T3DF: fibroblast-like cells differentiated from T3ES. The replicated data are calculated to the average value. High, Red; Middle, Black; Low, Green. (B) TSCOT gene expression during J1 mouse ES cell differentiation in vitro (GSE3749). Undifferentiated J1 ES cells (J1 ES 0 h) are maintained by LIF treatment. (C) TSCOT expression of non-SP and SP in mouse mammary gland (GSE5309). Ref: universal mouse reference (Stratagene).

FGF8, *FOXN1*, *IL22* and *FGF7*. In addition, *WNT5A*, *SHH*, *ISL1* and *GRHL3* (*Get1*) are also in the neighboring group with the expression pattern that is only transiently expressed. *Grhl3* is particularly interesting since knock out (KO) mouse skin show reduced *TSCOT* expression (see later).

The expression profile of *TSCOT* in the data from GSE3749 shows that the J1 ES cell line transiently expresses *TSCOT* when it is differentiated by removing leukemia inhibitory factor (LIF) (Fig. 4B).

When the data from various side populations were specifically searched for, SP of mouse mammary gland cells showed an increased *TSCOT* expression in the SP at a lower significance ($P = 0.0732$) (Fig. 4C). In some other SPs of mammary epithelium, studies did not reveal any significant difference between SP and non SP (data not shown).

From the results above, we concluded that *TSCOT* expression is initiated in differentiating ES cells and remained in some tissue committed SP stem cells.

TSCOT expression is independent of FOXN1 but depend on IRF6 and GRHL3

Because FOXN1 was considered as a putative TEC key tran-

scription factor, we searched for *TSCOT* expression in the remaining thymic rudiment of nude mouse. In RNA prepared from several tissues, *TSCOT* expression was found in the thymic rudiment of nude mice (Fig. 5A). RNA samples that were not treated with reverse transcriptase did not generate any bands (data not shown). This result is consistent with the conclusion derived from the gene profiling analyses.

In order to find the putative regulatory factors, differential *TSCOT* expressions were also examined in the skins of mouse lines with various mutations (Figs. 5B and 5C). In IRF6 KO mouse (GSE5800), *TSCOT* expression had reduced along with, *HoxC13*, *Fgf7*, *Tbx1*, and *Hoxa3* while *Notch3*, *Foxn1*, *Grhl3*, and *Wnt4* had increased. The opposite expression profiles of *TSCOT* and *Foxn1* in IRF6 KO skin suggest that these genes are independently regulated (Fig. 5B). It is interesting to note that the binding sites of IRF6 are located in the regulatory regions of *Grhl3* (Botti et al., 2011; de la Garza et al., 2012). More interestingly, *TSCOT* expression is also reduced in the skin of GRHL3 KO mice (GSE7381) (Fig. 5C). To investigate the actual involvement of those transcription factors will require more investigation. The effects of various cytokines for the *TSCOT* expression in the human epidermal keratinocytes (GSE7216) can also be visualized in Figure 5D. *TSCOT* expression is down regulated by IL1b, IL22 and IL24, but not by KGF, IFN γ , IL19, IL20 and IL26d (Fig. 5D) in the keratinocytes.

These results suggest a regulatory mechanism for *TSCOT* expression by the transcription factors, such as IRF6 and GRHL3, and by cytokines, but not by FOXN1.

Is TSCOT a tumor suppressor?

We also researched *TSCOT* expression in the lung development. Human fetal lungs at various stages between 54-154 days (GSE14334) are clustered in Fig. 6A. The genes that are expressed in a similar way to those of *TSCOT* are *GRHL3*, *FOXN1*, *PAX9*, and *CRIP3*. Those genes are known to be upregulated during the fetal lung developmental process (Kho et al., 2010). Therefore, it is likely that those transcription factors are involved in the positive regulator of *TSCOT* in the lung. The general patterns of *IRF6*, *SHH*, *ISL1*, and *SOX2* are downregulated during lung development, the opposite pattern to that of *TSCOT*. This suggests that those genes are potentially involved in the negative regulation of *TSCOT* in lung development.

In Fig. 6B, the cluster analysis of 20 genes coexpressed in the same fashion as *TSCOT* is shown in Table 3. To our surprise, *TSCOT* expression is clearly missing in three types of lung cancers, suggesting *TSCOT* may function as a tumor suppressor for lung cancer. The top genes clustered for the similar expression profiles are listed in Table III. As shown, many of the genes show possible tumor suppressor phenotypes (references in Table 3).

DISCUSSION

Using bioinformatics approaches and conventional molecular and cellular methods, we showed that *TSCOT* expression is turned on in TEC at the stem cell stage, and even prior to commitment of TEC lineages. In addition, we identified putative regulatory transcription factors and cytokines during thymus, skin and lung development.

TSCOT expression is turned on early thymic organogenesis and some other epithelial tissues

During last several years, gene expression profiling at the ge-

Table 3. List of genes down-regulated along with TSCOT during lung cancer development

Gene name*	Full name	Relation with cancer	Reference
<i>CLDN18</i>	Claudin-18	CLDN18 splice variant 2 is frequent Ectopic activation in pancreatic, Esophageal, ovarian, and lung tumors	Sahin et al., 2008
<i>CRTAC1</i>	Cartilage acidic protein 1	Copy number alteration in <i>CRTAC1</i> gene have been observed in neurofibromatosis Type 1-associated glomus tumors	Brems et al., 2009
<i>CYP4B1</i>	Cytochrome P450, Family 4, Subfamily B, Polypeptide 1	High expression of <i>CYP4B1</i> increases the risk of bladder tumor by activation of carcinogenic aromatic amines	Imaoka et al., 2000
<i>GKN2</i>	Gastrokine-2	Gastrointestinal tract specific gene <i>GKN2</i> might inhibit gastric cancer growth in a TFF1 dependent manner	Chu et al., 2012
<i>LRRK2</i>	Leucine-rich repeat serine	<i>LRRK2</i> G2019S mutations are associated with an increased cancer risk in Parkinson's disease	Saunders-Pullman et al., 2010
<i>SUSD2</i>	Sushi domain-containing protein 2	<i>SUSD2</i> increases the invasion of breast cancer cells and contributes to a potential immune evasion	Watson et al., 2013

*Gene names are listed in alphabetical order

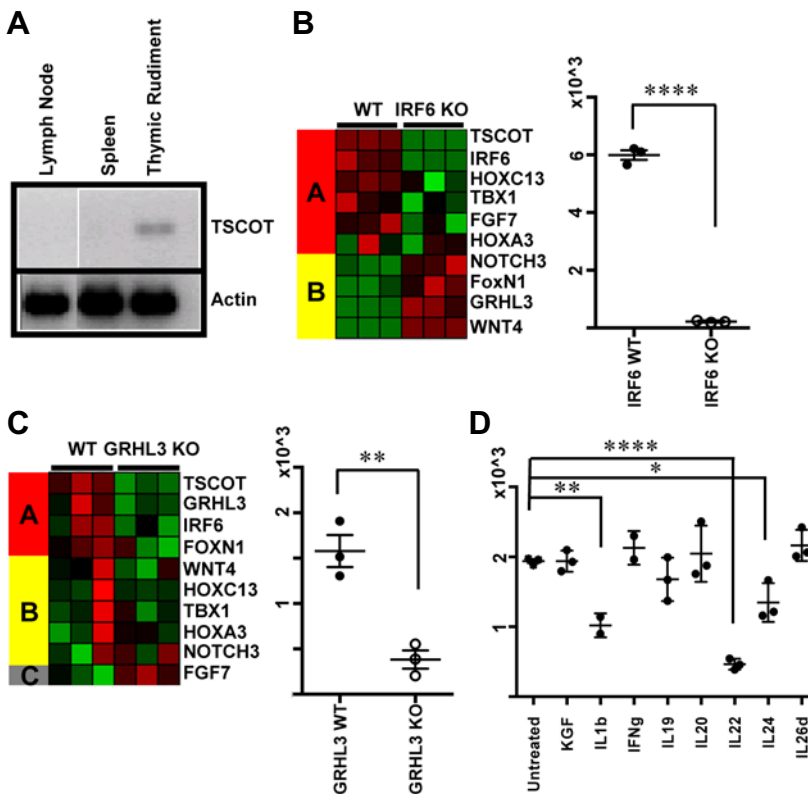


Fig. 5. Tissue specific expression of TSCOT reveals Foxn1 independency. (A) RT-PCR analysis of adult nude tissue. (B) Gene expression profiles from embryonic day 17.5 IRF6 KO and wild type mouse skin (GSE5800). Right panel: Comparison of TSCOT expression from skin between IRF6 KO mice and wild-type (P value < 0.0001****). (C) Gene expression profiles from embryonic day 18 GRHL3 KO and wild type mouse skin (GSE7381). Right panel: Comparison of TSCOT expression from skin between GRHL3 KO mice and wild-type (P value: 0.0042**). High, Red; Middle, Black; Low, Green. (D) TSCOT expression changes in human epidermal keratinocytes after treatment of KGF and various cytokines (GSE7216). Significant changes are compared to untreated cells (P < 0.0001****, P: 0.0025**, P: 0.0217*). Y axis are arbitrary units of process data.

nome scale are accumulated in the databases and accessible to the public. We took advantage of this advancement for the study of thymic organogenesis and TEC lineage differentiation using TSCOT as a lineage cell type specific marker and other known genes. From this approach, we learned that our previous studies are verified and we can get a lot more information than conventional experiments that are difficult to perform due

to the limitation of small numbers of cells present in the actual organ.

In our previous studies, we asserted that TSCOT is TEC lineage specific and expressed in the cTEC and bipotent pTEC (Ahn et al., 2008; Kim et al., 2000; Park et al., 2013). Tissue specificity in thymus and expression in the limited TEC lineages are verified by the genome scale data analysis of expression

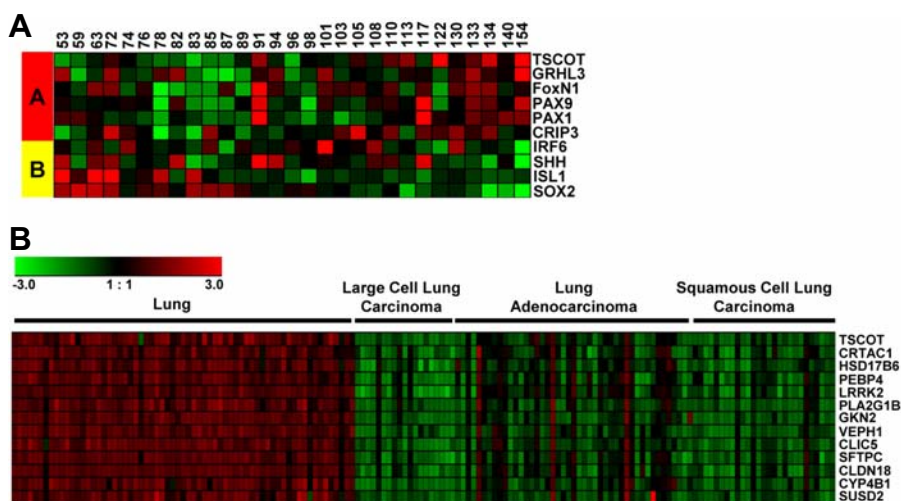


Fig. 6. Coexpression patterns in human lung development and lung cancer. (A) Gene expression change during lung development of human fetus (GSE14334). The numbers on the top indicate the number of days post conception. Replicated data are calculated to the average value. (B) A gene expression comparison between normal lung tissue and various lung tumor tissues from adult human (GSE19188). The gene expression values are normalized as 3.0 to -3.0 in the GENESIS program. High, Red; Middle, Black; Low, Green.

profiling (Figs. 1, 4, and 5). Furthermore, we learned that more tissues such as skin and lung also express *TSCOT*. The transcription factors involved in the thymic organogenesis may be also involved in skin and lung development (Figs. 1A-1C, 5B, 5C, and 6A). In addition, kinetic profiling of expression of *TSCOT* during cTEC lineage development has also been verified (Fig. 1C). *TSCOT* expression is highest in the youngest cTEC as we described earlier (Kim et al., 2000). *TSCOT* expression in mTEC^{lo} provides an interpretation that transitional cells found in the postnatal TEC preparation with UEA-1⁺CDR1^{lo} cells (Fig. 1D) are most likely the same kind. Earlier findings of sTECs in the medullary islet (Rodewald et al., 2001) and in the cortical medullary junction (Ahn et al., 2008) are consistent with the idea that TEC stem cells may overlap or share early mTEC features.

To further investigate cells at earlier stages than pTEC expression, we first utilized a functional SP analysis and showed that SP of fetal TEC preparation expresses *TSCOT* (Fig. 3). We like to call those cells sTEC for SP and stem TECs. In the SP of other type of tissues, such as mouse mammary glands, SP showed a slightly higher *TSCOT* expression than non SP (Fig. 4C). Our search for *TSCOT* expression in ES cells produced interesting results that *TSCOT* is induced in the differentiating embryonic bodies or ES cell cultures without LIF. *TSCOT* expression is off when the cells are differentiated into pancreatic epithelium or fibroblasts (Fig. 4A), and in the endothelial lineage (Fig. 2C). These results support the idea that *TSCOT* is expressed at the stem cell stage during certain organogenesis.

Given the concept that *TSCOT* is expressed in sTEC, its expressions in the skin and lung are not very surprising. They all express epithelial markers such as EpCAM and Keratins. There are cases that these cell lineages are actually interconvertible under certain circumstances. The stem cell preparation from TEC can be differentiated into skin type keratinocyte (Bonfanti et al., 2010) and thymic epithelium of nude mouse has shown to have lung epithelial morphologies (Dooley et al., 2005). This phenomenon can be interpreted as that of a reprogramming of gene expression, transforming stem cells which are committed to one organ type, into another at the level of master gene expression.

A schematic model in Figure 7 summarizes the findings of the expression profile during organogenesis. *TSCOT* expres-

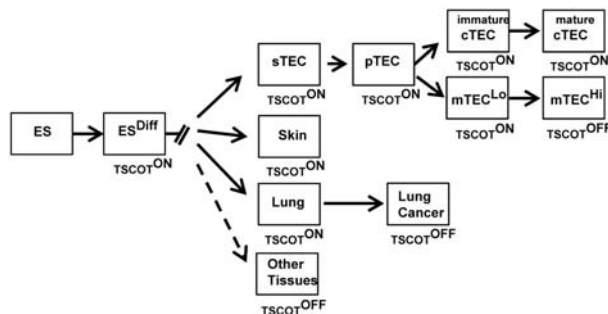


Fig. 7. Summary model of *TSCOT* expression profile during organogenesis. *TSCOT* expressions are indicated at the bottom of each text box. ES, embryonic stem cells; ES^{Diff}, differentiating ES cells.

sion turned on in early uncommitted ES cells may be maintained in thymus, skin, and lung. In other organs, *TSCOT* expression is now turned off. The stem cell for the thymic organogenesis and the precursor TEC (pTEC) maintains *TSCOT* expression. The cTEC and mTEC^{lo} cells are cells that express *TSCOT*, and mature mTEC^{hi} cells lose *TSCOT* expression. In lung, *TSCOT* expression is turned on but tumorigenesis will turn off *TSCOT* expression.

Modulation of *TSCOT* gene expression was revealed by gene expression profiling

By cluster analyses with selected transcription and soluble factors that are shown to be involved during the thymic organogenesis, we were able to identify multiple putative positive or negative regulators. IRF6 and GRHL3 are putative positive regulators for *TSCOT* expression in the skin (Figs. 5B and 5C). It has been reported that GRHL3 is located downstream of IRF6 in human keratinocytes (Botti et al., 2011; de la Garza et al., 2012; Malik et al., 2010). *TSCOT* expression potentially is regulated by IL1b, IL22, and IL24 in negative fashion (Fig. 5D). These results suggest that *TSCOT* is controlled by IRF6 and GRHL3, and IL1b, IL22, and IL24 in human keratinocyte. In contrast, in human fetal lung development, both *TSCOT* and *GRHL3* expressions are upregulated together while *IRF6* ex-

pression is downregulated (Fig. 6A). These phenomena suggest the complex network of expression regulation and/or cross checking regulation of genes during different epithelial tissue development.

TSCOT may be a new member of the tumor suppressors

Besides the structural features of a transporter that appeared containing primary amino acid sequences (Kim et al., 2000), it is still unclear what biological and biochemical functions that TSCOT plays. TSCOT is a member of Slc46A, and another member, Slc46A1, has been characterized as a proton coupled folate transporter (Diop-Bove et al., 2013). The heavily hydrophobic nature of the TSCOT amino acid composition and a simple twelve membrane spanning feature, with the presence of a central inner loop in the absence of ATP binding domain, suggests that TSCOT may transport small hydrophobic molecules. We proposed earlier that it may function in the survival of TECs based on the expression (Kim et al., 2000).

It is exciting to find that TSCOT expression in the lung disappear in three types of lung cancers, large lung cell carcinoma, lung adenocarcinoma, and squamous lung carcinoma (Fig. 6B). This expression strongly implies TSCOT may function as a type of tumor suppressor. This supports the fact that TSCOT also function in the same way for the genetic type of cervical cancer susceptibility proposed (Engelmark et al., 2006; 2008). In fact, the Human TSCOT locus (9q32) was mapped to the susceptibility of cervical cancer through a SNP polymorphism study (Engel mark et al., 2006; 2008). It may function as a necessary component to maintain normal epithelium. When it is missing in lung epithelium, carcinogenesis progresses without hindrance. Other genes expressed in a similar fashion (Fig. 6) also show the functionality in tumor suppressors as described in Table III.

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