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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 cotransporter (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.

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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 (TTACTCAAAGTGATGCTGGACTGG), 1C12 B2 (CCGAGGGTTCCTTGGTACATTC), and the EGFP locus: EGFP-F (GCCACAAGTTCAGCGTGTCC), EGFP-R (GCTTCTGTTGGGGTCTTTGC), 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	*AIRE*	208090_s_at	NM_000658
	BMP4	211518_s_at	D30751
	CD248	219025_at	NM_020404
	CLDN18	214135_at	BE551219
	CLIC5	219866_at	NM_016929
	CRIP3	235720_at	AI042209
	CRTAC1	221204_s_at	NM_018058
	CYP4B1	1555497_a_at	AY151049
	EYA1	214608_s_at	AJ000098
	FGF7	205782_at	NM_002009
	FGF8	208449_s_at	NM_006119
	FOXG1	206018_at	NM_005249
	FOXN1	207683_at	NM_003593
	GKN2	238222_at	AI821357
	GRHL3	232116_at	AL137763
	HOXA3	208604_s_at	NM_030661
	HOXC13	219832_s_at	NM_017410
	HSD17B6	205700_at	NM_003725
	IL22	221165_s_at	NM_020525
	IL6	205207_at	NM_000600
	IL7	206693_at	NM_000880
	IRF6	1552478_a_at	NM_006147
	ISL1	206104_at	NM_002202
	LIF	205266_at	NM_002309
	LRRK2	229584_at	AK026776
	NOTCH3	203238_s_at	NM_000435
	OSM	230170_at	AI079327
	PAX1	1553492_a_at	NM_006192
	PAX9	207059_at	NM_006194
	PEBP4	227848_at	AI218954
	PLA2G1B	206311_s_at	NM_000928
	SFTPC	215454_x_at	AI831055
	SHH	207586_at	NM_000193
	SIX1	205817_at	NM_005982
	SLC46A2	223816_at	AF242557
	SOX2	228038_at	AI669815
	SUSD2	234310_s_at	AK026431
	TBX2	207662_at	NM_005992
	VEPH1	232122_s_at	AK022666
	WNT4	208606_s_at	NM_030761
	WNT5A	205990_s_at	NM_003392
	WNT5B	221029_s_at	NM_030775
GPL1261	*Aire*	1419241_a_at	NM_009646
	Bcl2	1422938_at	NM_009741
	Bmp4	1422912_at	NM_007554
	CD248	1417439_at	NM_054042
	Crip3	1451410_a_at	AF367970
	Dab2	1420498_a_at	NM_023118
	Dkk3	1417312_at	AK004853
	Eya1	1421727_at	NM_010164
	Fgf7	1422243_at	NM_008008
	Fgf8	1451882_a_at	U18673
	FoxG1	1418357_at	NM_008241
	FoxN1	1450508_at	NM_008238
	Gas1	1416855_at	BB550400
	Hoxa3	1452421_at	BB496114
	HoxC13	1425874_at	AF193796
	IL6	1450297_at	NM_031168
	IL7	1422080_at	NM_008371
	Irf6	1418301_at	NM_016851
	Isl1	1422720_at	BQ176915
	Lif	1450160_at	AF065917
	Ly75	1449328_at	NM_013825
	Meis1	1443260_at	BB055155
	Notch3	1421964_at	NM_008716
	Osm	1438767_at	BB237825
	Pax1	1449359_at	NM_008780
	Pax9	1421246_at	BC005794
	Pbx1	1449542_at	NM_008783
	Psmb11	1453150_at	BG069341
	Shh	1436869_at	AV304616
	Six1	1427277_at	BB137929
	Six4	1456862_at	AI893638
	Slc46a2	1423476_at	BB329435
	Sox2	1416967_at	U31967
	Tbx1	1425779_a_at	AF326960
	Tert	1450254_at	NM_009354
	Wnt4	1450782_at	NM_009523
	Wnt5a	1436791_at	BB067079
	Wnt5b	1422602_a_at	NM_009525
GPL2987	*FOXN1*	hCG31797.3	NM_003593.2
	HOXA3	hCG1640627.4	NM_153632.1, NM_030661.3, NM_153631.1
	PAX9	hCG20991.2	NM_006194.1
	SLC46A2	hCG29190.4	NM_033051.2
GPL8217	*FOXN1*	HSG00201177 (ROSETTAGENE MODEL_ID)	NM_006015
	HOXA3	HSG00314123 (ROSETTAGENE MODEL_ID)	NM_002309
	PAX9	HSG00282340 (ROSETTAGENE MODEL_ID)	NM_030775
	SLC46A2	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

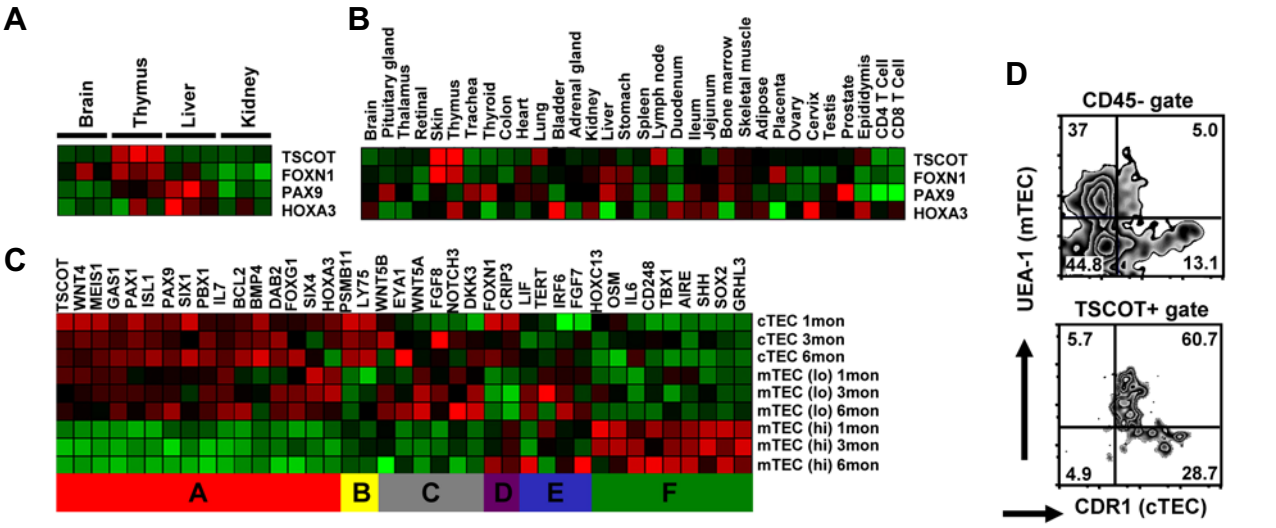

Fig. 1. Tissue and cell type specific TSCOT expression profiling. (A) Clustering of *TSCOT* with three other genes, *FOXN1*, *PAX9*, *HOXA3,* expression in human fetal tissues (GSE7905). (B) Expression in human adult tissues (GSE14938). (C) Gene expression during TEC development. cTEC and mTEC from adult mice are analyzed (GSE 56928). mTEC (lo): $CD80^{lo}$ and $MHC\text{-}II^{lo}$, mTEC (hi): $CD80^{Hi}$ and $MHC\text{-}II^{Hi}$. 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 (FACSAriaII). For multicolor staining with SP analysis, cells were prestained with selected antibodies including homemade 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-CGATTCCATGTGCCCCATTG-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 $MgCl_2$ 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 $MgCl_2$, 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
AIRE	Autoimmune regulator	Regulate mTEC development and differentiation, Transcription factor	Gordon and Manley, 2011; Sun et al., 2013	
BCL2	Growth Arrest-Specific 1	Antiapoptotic gene	Wong et al., 2014	
BMP4	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)
CD248	CD248 Molecule, Endosialin	Required for postnatal thymic growth and regeneration following infection-dependent thymic atrophy	Liu et al., 2014	
CRIP3 (TLP)	Cystein-Rich Protein 3 (Thymus Lim Protein)	Appears to have a role in normal thymus development	Kirchner et al., 2001	
DAB2	Mitogen-Responsive Phosphoprotein, Homolog	Wnt-inhibitors, Control proliferation and differentiation of stem cells into lineage-restricted cells	Wong et al., 2014	
DKK3	Dickkopf WNT Signaling Pathway Inhibitor 3	Wnt-inhibitors, Control proliferation and differentiation of stem cells into lineage-restricted cells	Wong et al., 2014	
EYA1	Eyes absent 1 homolog	Necessary for 3rd pouch development	Wei and Condie, 2011; Gordon and Manley, 2011	
FGF7 (KGF)	Keratinocyte growth factor	Induces mature and immature TECs and promotes differentiation of immature TECs	Rossi et al., 2006	
FGF8	Fibroblast growth factor 8	Indirectly influence TECs by regulating neural crest cells survival and differ entiation, relate to early pouch formation	Gordon and Manley, 2011; Sun et al., 2013	
FOXG1	Forkhead Box G1	May play a role in the regulation of TEC differentiation during fetal and postnatal stages, Transcription factor	Wei and Condie, 2011	
FOXN1	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	
GAS1	Growth Arrest-Specific 1	Cell-cycle suppressor gene	Wong et al., 2014	
GRHL3 (Get-1)	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**)
HOXA3	Homeobox A3	Early pouch patterning and initial organ formation, Transcription factor	Manley and Capecchi, 1995; Su et al., 2001; Gordon and Manley, 2011	
HOXC13	Homeobox C13	Mediates transcriptional regulation of Foxn1, Transcription factor	Potter et al., 2010	
IL22	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****)
IL6	Interleukin 6	Associated with thymic involution	Chinn et al., 2012	
IL7	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
IRF6	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****)
ISL1	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	
LIF	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)
LY75 (NLDC205, DEC205)	Lymphocyte antigen 75	Contribute to antigen presentation, Marker of cTEC in adult thymus	Jiang et al., 1995; Shakib et al., 2009	
MEIS1	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	
NOTCH3	Notch homolog protein 3	Regulate murine T cell differentiation and leukemogenesis	Bellavia et al., 2008	
OSM	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	
PAX1	Paired Box 1	Early pouch formation and parathyroid development, minor role in thymus size, Transcription factor	Wallin et al., 1996; Gordon and Manley, 2011	
PAX9	Paired Box 9	Pouch and initial organ formation, TEC differentiation, Transcription factor	Hetzer-Egger et al., 2002; Gordon and Manley, 2011	
PBX1	Pre-B-cell leukemia homeobox	Required for embryonic thymic organogenesis, Transcription factor	Hirayama et al., 2014	
PSMB11 (β5t)	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	
SHH	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)
SIX1/4	Sine oculis-related homeobox 1/4	Necessary for 3rd pouch development, Transcription factor	Wei and Condie, 2011; Gordon and Manley, 2011	
SOX2	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**)
TBX1	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
TERT	Telomerase Reverse Transcriptase	Telomerase reverse transcriptase	Wong et al., 2014	
WNT4	Wingless-type MMTV integration site family, member 4	Controls thymopoiesis and thymus size by regulating TEC, thymocyte and their progenitor proliferation, regulate Foxn1 expression in TECs	Sun et al., 2013	
WNT5A	Wingless-type MMTV integration site family, 5A	Regulate the survival of αβ lineage thymocytes, regulator of cell growth in hematopoietic tissue	Liang et al., 2007	
WNT5B	Wingless-type MMTV integration site family, 5B	Produced by TECs and thymocytes, regulate Foxn1 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* (β5t) and *Ly75* (NLDC202/DEC205) that are genuine cTEC-specific genes. Group C (*Wint5a, 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 *Grhl3*. 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 expressed 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

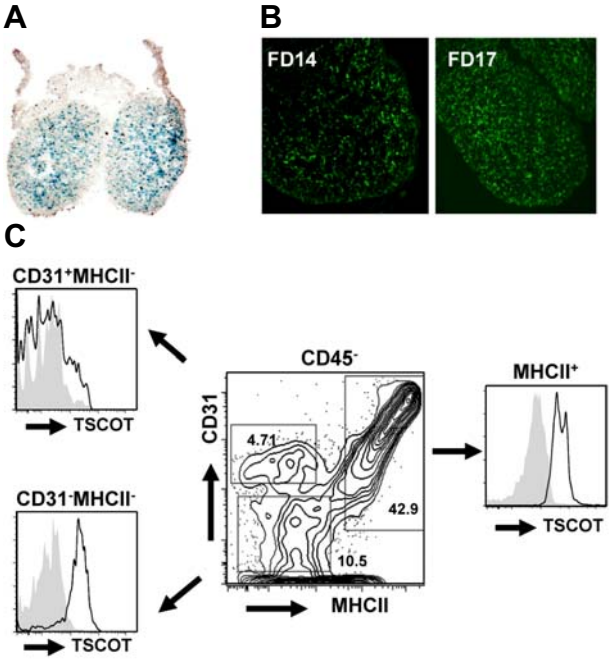

Fig. 2. TSCOT is expressed in fetal TEC committed cells. (A) LacZ expression in the FD14 thymus of TDLacZ mouse. (B) EGFP expression in the fetal 3.1T-EGFP transgenic thymus. (C) Flow cytometric analysis of fetal TEC preparation for TEC and endothelial lineages. CD31 is for endothelial cells, MHCII is 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

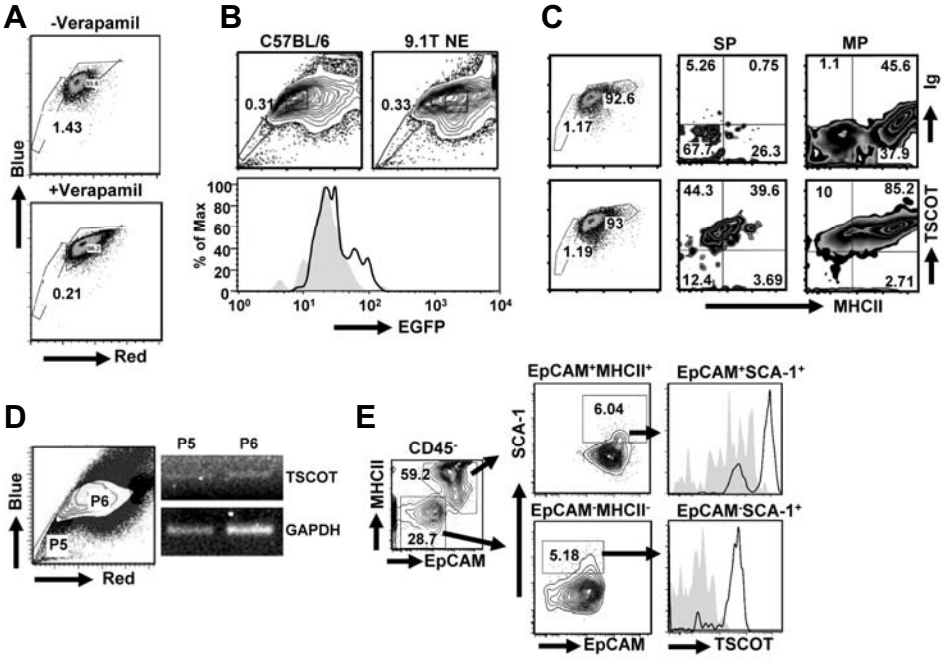

Fig. 3. SP analysis of TEC preparation. (A) SP analysis of fetal TEC preparation. Verapamil was included for blocking ABC transporter function during staining with Hoechst 33342. (B) EGFP expression of SP in the fetal TEC preparation from 9.1T-NE. EGFP levels are compared with the SP from C57BL/6. (C) A multicolor analysis of SP with prestained 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.

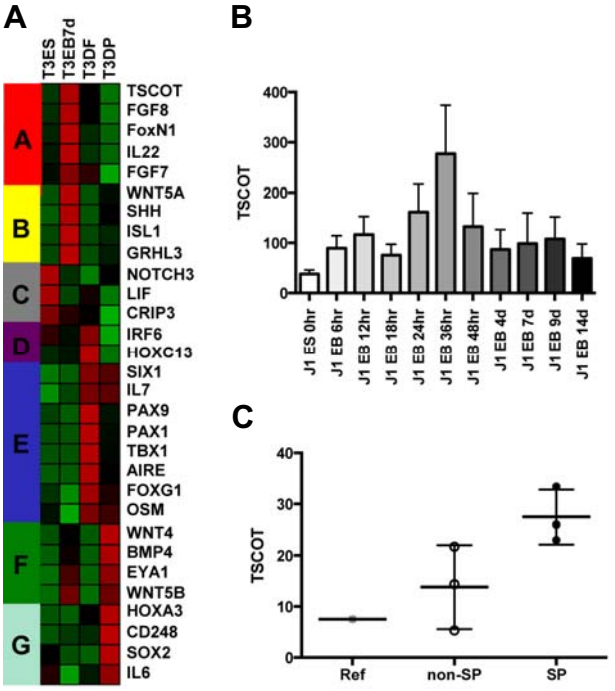

Fig. 4. Gene clustering analysis of ES and differentiating ES cells. (A) Gene expression level of pancreatic islet-like cell clusters and fibroblast-like cells derived from human T3 embryonic stem cells (GSE14503 and GSE9440). T3ES: human T3 embryonic stem cell, T3EB7d: day 7 embryonic body derived from 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 transcription 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, IFNγ, 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
CLDN18	Claudin-18	CLDN18 splice variant 2 is frequent Ectopic activation in pancreatic, Esophageal, ovarian, and lung tumors	Sahin et al., 2008
CRTAC1	Cartilage acidic protein 1	Copy number alteration in CRTAC1 gene have been observed in neurofibromatosis Type 1-associated glomus tumors	Brems et al., 2009
CYP4B1	Cytochrome P450, Family 4, Subfamily B, Polypeptide 1	High expression of CYP4B1 increases the risk of bladder tumor by activation of carcinogenic aromatic amines	Imaoka et al., 2000
GKN2	Gastrokine-2	Gastrointestinal tract specific gene GKN2 might inhibit gastric cancer growth in a TFF1 dependent manner	Chu et al., 2012
LRRK2	Leucine-rich repeat serine	LRRK2 G2019S mutations are associated with an increased cancer risk in Pakinson's disease	Saunders-Pullman et al., 2010
SUSD2	Sushi domain-containing protein 2	SUSD2 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

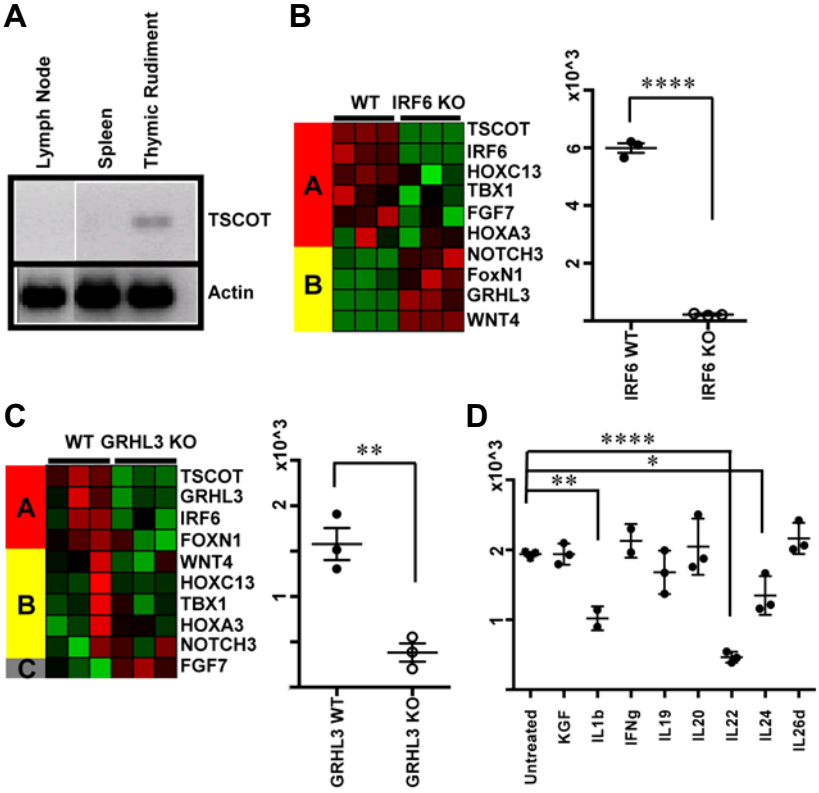

Fig. 5. Tissue specific expression of TSCOT reveals Foxn1 independency. (A) RT-PCR analysis of adult nude tissue. (B) Gene expression profiles from embryonic day 17.5 IRF6 KO and wild type mouse skin (GSE5800). Right panel: Comparison of TSCOT expression from skin between IRF6 KO mice and wild-type (P 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 arbiturary 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

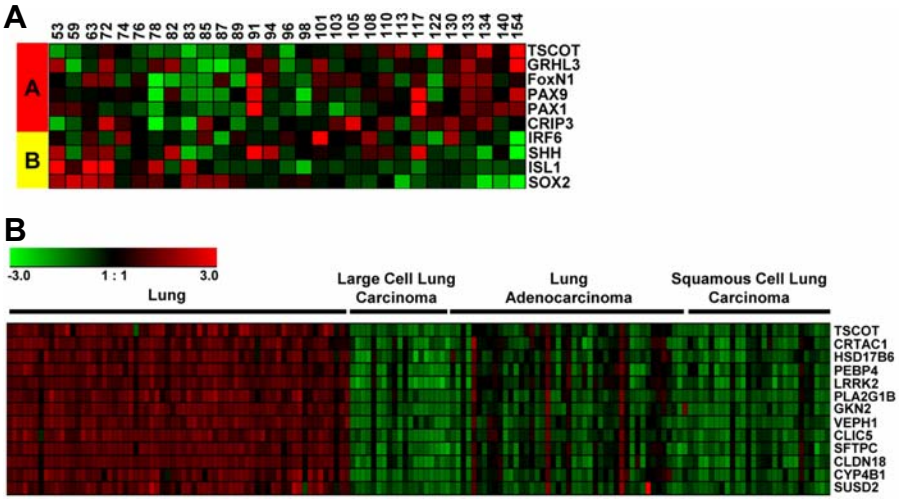

Fig. 6. Coexpression patterns in human lung development and lung cancer. (A) Gene expression change during lung development of human fetus (GSE14334). The numbers on the top indicate the number of days post conception. Replicated data are calculated to the average value. (B) A gene expression comparison 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 reprograming 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* expression 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.

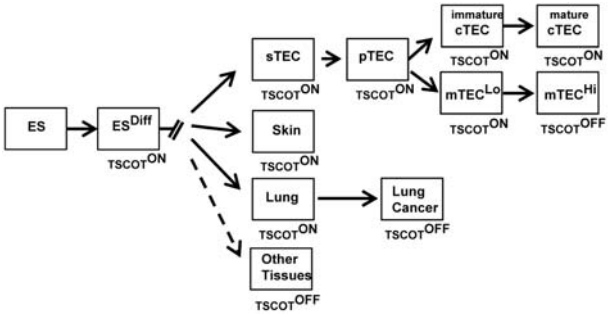

Fig. 7. Summary model of TSCOT expression profile during organogenesis. TSCOT expressions are indicated at the bottom of each text box. ES, embryonic stem cells; ES^{Diff}, differentiating ES cells.

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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