Analysis of uterine gene expression in interleukin-15 knockout mice reveals uterine natural killer cells do not play a major role in decidualization and associated angiogenesis

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Abstract

During decidualization, uterine natural killer (uNK) cells are the most abundant immune cell types found in the uterus. Although it is well known that they play key roles in spiral arteriole modification and the maintenance of decidual integrity seen after mid-pregnancy, their roles in the differentiation of decidual cells and accompanying angiogenesis during the process of decidualization is less well characterized. To address this, we used whole-genome Illumina BeadChip analysis to compare the gene expression profiles in implantation segments of the uterus during decidualization on day 7.5 of pregnancy between wild-type and uNK cell-deficient (interleukin-15-knockout) mice. We found almost 300 differentially expressed genes and verified the differential expression of ~60 using quantitative RT-PCR. Notably, there was a lack of differential expression of genes involved in decidualization and angiogenesis and this was also verified by quantitative RT-PCR. Similar endothelial cell densities and proliferation indices were also found in the endometrium between the implantation site tissues of wild-type and knockout mice undergoing decidualization. Overall, the results of this study reveal that uNK cells likely do not play a major role in decidualization and accompanying angiogenesis during implantation. In addition, the study identifies a large number of genes whose expression in implantation-site uterine tissue during decidualization depends on interleukin-15 expression in mice.

Reproduction (2012) **143** 359–375

Introduction

Decidualization, the differentiation of the endometrium into the decidua, is a key process during implantation of the conceptus in rodents and humans and has been covered by a number of reviews (Abrahamsohn & Zorn 1993, Dunn *et al.* 2003, Gellersen *et al.* 2007, Herington *et al.* 2009, Ramathal *et al.* 2010). Briefly, in mice, the decidual tissue provides the nutritive environment for ~5 days in which the embryo and placenta develop. After mid-pregnancy, the functional placenta is formed and takes over providing the nutrients to the fetus. Several changes occur in the uterus during decidualization, but the hallmark is the rapid proliferation and then transdifferentiation of fibroblast-like endometrial stromal cells into the epithelial-like decidual cells, usually called decidual cell differentiation. In the mouse, this is dependent on the actions of several hormones including progesterone and bone morphogenetic protein 2 (BMP2; Lee *et al.* 2007) and is accompanied by an increase in the expression of decidual markers such as liver/bone/kidney alkaline phosphatase (*Akp1*; Herington *et al.* 2009) and prolactin family 8, subfamily a, member 2

(*Prl8a2*; Bany & Cross 2006), and a massive increase in uterine weight. To support this tissue growth, another important process during decidualization of the endometrium is angiogenesis or neo-angiogenesis (Torrey *et al.* 2007, Laws *et al.* 2008, Demir *et al.* 2010). Critical to this is the endometrial expression of several genes such as vascular endothelial growth factor A (*Vegfa*; Chakraborty *et al.* 1995, Halder *et al.* 2000), prostaglandin endoperoxide synthase 2 (*Ptgs2*) plus angiopoietin 2 (*Angpt2*) (Matsumoto *et al.* 2002), and gap junction protein alpha 1 (*Gja1*; Laws *et al.* 2008).

The most abundant immune cell types in the uterus during decidualization are uterine natural killer (uNK) cells. Current dogma for mice is that there are two sources of uNK cells in the uterus during implantation (Zhang *et al.* 2009, 2011). One population of uNK cells are dolichos biflorus agglutinin (DBA) lectin-positive (DBA⁺) and have granules that stain positive using periodic acid Schiff staining (PAS⁺). The DBA⁺PAS⁺ uNK cells are believed to be derived from circulating lymphocyte progenitor cells, which upon entering the uterus as immature non-granulated uNK cells, begin

expressing DBA lectin and undergo maturation into large granulated NK cells. The other source of uNK cells in the uterus comes from the resident uNK cells which are DBA lectin-negative (DBA⁻) but are PAS⁺. A great deal of work has been conducted on the functions of uNK cells in mice and has involved the use of several genetic models, including interleukin-15 knockout (*Il15*^{-/-}) mice, where uNK cells are absent from the uterus during decidualization (Croy *et al.* 2003b). All of these studies conclusively showed that uNK cells play a key role in maintaining decidual integrity and the characteristic modification of the spiral arteries which is clearly seen only after mid-pregnancy. However, it should be noted that the uNK cell deficient *Il15*^{-/-} mice are fertile, have normal gestation times and comparable litter sizes to wild-type mice (Barber & Pollard 2003). However, although little is really known about the role of uNK cells in decidualization and accompanying angiogenesis, it has been speculated that they play a role in decidualization. For example, it has been speculated that uNK cells play a role in uterine angiogenesis because in humans and mice, they express *VEGFC* and *Vegfa* respectively (Wang *et al.* 2000, Lash *et al.* 2006). However, a clear role for uNK cells in decidual cell differentiation and angiogenesis during decidualization is currently not established.

The present study was conducted to more closely examine the potential aberrant expression of genes involved in decidual cell differentiation and angiogenesis in the mouse uterus during decidualization in *Il15*^{-/-} mice. The results support the hypothesis that uNK cells in mice do not play a major role in decidual cell differentiation as well as angiogenesis during decidualization. However, the microarray analysis reveals the identity of almost 300 genes whose expression is altered in the implantation areas of the uterus of *Il15*^{-/-} mice, some of which were investigated further.

Results

Microarray analysis

To determine the potential effect of uNK cell depletion on uterine gene expression during decidualization, we compared the global mRNA levels between RNA samples in day 7.5 implantation segment (IS) tissues from *Il15* wild-type (*Il15*^{+/+}) and knockout mice (*Il15*^{-/-}) using Illumina BeadChip microarray analysis. day 7.5 of pregnancy was chosen because by this day decidual growth is well underway and uNK cells are very abundant in IS tissue of the uterus (Herington & Bany 2007b). Of the 46 633 probes, signals for 14 148 were detected significantly above background in all samples. Analysis of these data suggested that 285 of these probes showed a significant difference in the level of hybridization signals between samples from *Il15*^{-/-} and *Il15*^{+/+} mice and could be assigned a gene symbol.

These probes represented the mRNAs for 267 different genes that were differentially expressed, with 150 and 117 having greater hybridization signals in IS tissue from *Il15*^{+/+} and *Il15*^{-/-} mice respectively. Genes listed in Tables 1 and 2 are those differentially expressed twofold or greater in the IS tissue of *Il15*^{+/+} and *Il15*^{-/-} mice respectively. Further analysis of the microarray data revealed that hybridization signals for 29 and 7 probes, respectively, were found in IS tissues of only *Il15*^{+/+} (Table 3) or *Il15*^{-/-} (Table 4) mice. These represented probes representing 26 and 7 genes respectively. Full annotation of all 321 probes representing differential expression of 300 genes is shown in Supplementary Table 1, see section on supplementary data given at the end of this article. Finally, the hypogeometric tests for gene ontology (GO) terms of the list of all differentially expressed genes revealed significant over- and under-representation of GO terms for molecular function (Supplementary Table 2, see section on supplementary data given at the end of this article). The top over-represented biological process was proteolysis and several immune cell-related processes were in the list.

Expression of genes involved in decidualization

Although it is clear that 'decidual integrity' depends on the presence of uNK cells (Ashkar *et al.* 2003, Monk *et al.* 2005), it has not been solidly established whether or not these cells play a role in decidual cell differentiation. The expression of many genes in rodent endometrial stromal cells is known to increase during decidual cell differentiation. Notably, in this study, the mRNA levels of two of the more commonly used decidual markers, *Prl8a2* and *Akpl*, were not different between the IS tissue of *Il15*^{+/+} compared with *Il15*^{-/-} mice. Two major regulators of decidualization are BMP2 and progesterone (Lee *et al.* 2007). The mRNA levels of *Bmp2* and progesterone receptor (*Pgr*) were not found to be differentially expressed in the microarray analysis. As shown in Fig. 1A, we verified the lack of differential mRNA levels for *Bmp2* as well as several BMP2 target genes in the uterus such as FK506 binding protein 3 (*Fkbp3*), 4 (*Fkbp4*), and 5 (*Fkbp5*). In addition, mechanistic targets of rapamycin (*Mtor/Frap1*), follistatin (*Fst*), and inhibitor of DNA binding 1 (*Id1*) (Lee *et al.* 2007) were not significantly ($P > 0.05$) different in IS tissue between *Il15*^{+/+} and *Il15*^{-/-} mice (Fig. 1A). In a similar fashion, we verified that *Pgr* mRNA levels did not differ in IS tissues between these mice (Fig. 1A). Finally, Kruppel-like factor 5 (*Klf5*) is expressed in the uterus (Watanabe *et al.* 1999) and is known to be involved in vascular remodeling and angiogenesis (Nagai *et al.* 2005). *Klf5* mRNA levels in the IS tissues of the uterus significantly ($P < 0.05$) increased compared with non-implantation segment (NIS) tissues on days 5.5–8.5 of pregnancy in *Il15*^{+/+} mice (Fig. 1B). Although the microarray analysis indicated differential expression of

Table 1 Genes whose steady-state mRNA levels are significantly greater in IS tissue from day 7.5 pregnant *IL15*^{+/+} mice compared with that of *IL15*^{-/-} mice by at least an average of twofold. Probe ID indicates Illumina Probe identification number and fold refers to average fold difference mRNA levels in IS tissue of *IL15*^{+/+} relative to *IL15*^{-/-} mice.

Gene symbol	Name	Fold	Probe ID
<i>Spp1</i>	Secreted phosphoprotein 1	11.931	2470609
<i>Gdpd3</i>	Glycerophosphodiester phosphodiesterase domain containing 3	6.423	6110292
<i>Ear11</i>	Eosinophil-associated, ribonuclease A family, member 11	5.938	2260292
<i>Tcrb-J</i>	T-cell receptor beta, joining region	5.040	103800086
<i>Rgs5</i>	Regulator of G-protein signaling 5	3.982	101410068
<i>Aldh1a3</i>	Aldehyde dehydrogenase family 1, subfamily A3	3.775	105700162
<i>Pdgfrl</i>	Platelet-derived growth factor receptor-like	3.749	870168
<i>Tnfrsf9</i>	Tumor necrosis factor receptor superfamily, member 9	3.138	2510400
<i>Csrnp1</i>	Cysteine-serine-rich nuclear protein 1	3.095	1230053
<i>Tpsab1</i>	Tryptase alpha/beta 1	3.095	1400347
<i>Pdpn</i>	Podoplanin	3.095	4640280
<i>Pstpip1</i>	Proline-serine-threonine phosphatase-interacting protein 1	2.921	3190156
<i>Degs2</i>	Degenerative spermatocyte homolog 2, lipid desaturase	2.915	1170056
<i>Rac2</i>	RAS-related C3 botulinum substrate 2	2.802	1580541
<i>Irf8</i>	Interferon regulatory factor 8	2.751	610161
<i>Necap2</i>	NECAP endocytosis associated 2	2.726	6200048
<i>Cldn15</i>	Claudin 15	2.682	4150270
<i>Selplg</i>	Selectin, platelet (p-selectin) ligand	2.651	1770167
<i>Fbln1</i>	Fibulin 1	2.585	2480059
<i>Sprr2d</i>	Small proline-rich protein 2D	2.579	1340458
<i>A430104N18Rik</i>	RIKEN cDNA A430104N18 gene	2.537	106510711
<i>Cinp</i>	Cyclin-dependent kinase 2 interacting protein	2.514	106770484
<i>Ncf4</i>	Neutrophil cytosolic factor 4	2.474	610164
<i>Tbc1d20</i>	TBC1 domain family, member 20	2.440	7000286
<i>Cnot7</i>	CCR4-NOT transcription complex, subunit 7	2.428	101570114
<i>Coro1a</i>	Coronin, actin binding protein 1A	2.417	3140609
<i>Gnl2</i>	Guanine nucleotide binding protein-like 2	2.406	6620022
<i>Myo1g</i>	Myosin IG	2.395	2970358
<i>Fbln2</i>	Fibulin 2	2.395	3440215
<i>Coro1a</i>	Coronin, actin binding protein 1A	2.367	3190020
<i>Lat2</i>	Linker for activation of T cells family, member 2	2.362	5340440
<i>Spink2</i>	Serine peptidase inhibitor, Kazal type 2	2.357	3990093
<i>Wrn</i>	Werner syndrome homolog	2.329	540692
<i>Cd47</i>	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	2.303	103390731
<i>9130230L23Rik</i>	RIKEN cDNA 9130230L23 gene	2.271	2190452
<i>Pnpla3</i>	Patatin-like phospholipase domain containing 3	2.266	5050551
<i>Rab44</i>	RAB44, member RAS oncogene family	2.240	102230139
<i>Farsa</i>	Phenylalanyl-tRNA synthetase, alpha subunit	2.235	2650593
<i>Ocln1</i>	Occludin/ELL domain containing 1	2.204	2470088
<i>Anxa8</i>	Annexin A8	2.194	4780022
<i>Akr1b8</i>	Aldo-keto reductase family 1, member B8	2.184	3120288
<i>Rap1gap</i>	Rap1 GTPase-activating protein	2.184	101850672
<i>Retnla</i>	Resistin-like alpha	2.168	3840138
<i>Pfpl</i>	Pore forming protein-like	2.149	6450670
<i>Fam83g</i>	Family with sequence similarity 83, member G	2.144	103130735
<i>Col5a2</i>	Collagen, type V, alpha 2	2.139	104280100
<i>Doxl2</i>	Diamine oxidase-like protein 2	2.134	104780056
<i>Ccdc114</i>	Coiled-coil domain containing 114	2.129	104610181
<i>Pcsk5</i>	Proprotein convertase subtilisin/kexin type 5	2.109	5720600
<i>Emid2</i>	EMI domain containing 2	2.099	360400
<i>Serhl</i>	Serine hydrolase-like	2.090	2470605
<i>Ciapi1</i>	Cytokine-induced apoptosis inhibitor 1	2.080	2900114
<i>Selplg</i>	Selectin, platelet (p-selectin) ligand	2.056	101470563
<i>Sft2d3</i>	SFT2 domain containing 3	2.051	103060670
<i>Oxsr1</i>	Oxidative-stress responsive 1	2.047	1500025
<i>Alox5</i>	Arachidonate 5-lipoxygenase	2.037	105340280
<i>Adamts7</i>	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 7	2.023	3360368
<i>Gabbr1</i>	Gamma-aminobutyric acid (GABA) B receptor, 1	2.000	101780504

Table 2 Genes whose steady-state mRNA levels are significantly less in IS tissue from day 7.5 *Il15*^{-/-} pregnant mice compared with that of *Il15*^{+/+} mice by twofold or greater. Probe ID denotes Illumina Probe identification number and fold indicates average fold difference mRNA levels in IS tissue of *Il15*^{+/+} relative to *Il15*^{-/-} mice.

Gene symbol	Name	Fold	Probe ID
<i>Igkc</i>	Immunoglobulin (Ig) and T-cell receptor	11.628	1230347
<i>Igkc</i>	Immunoglobulin kappa constant	10.526	6370309
<i>Igh-VJ558</i>	Immunoglobulin heavy chain (J558 family)	9.709	60025
<i>Ndufb10</i>	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10	8.850	5900576
<i>Igh-VJ558</i>	Immunoglobulin heavy chain (J558 family)	8.333	1690184
<i>Man2b1</i>	Mannosidase 2, alpha B1	6.410	106520575
<i>Rassf8</i>	Ras association (RalGDS/AF-6) domain family (N-terminal) member 8	4.505	105690053
<i>Glrx</i>	Glutaredoxin	4.444	3520471
<i>Scoc</i>	Short coiled-coil protein	3.937	2230053
<i>Prdx2</i>	Peroxiredoxin 2	3.891	4010619
<i>Arl3</i>	ADP-ribosylation factor-like 3	3.817	5690725
<i>Trmt1</i>	TRM1 tRNA methyltransferase 1 homolog	3.759	103780280
<i>Pla2g12a</i>	Phospholipase A2, group X1A	3.636	103390286
<i>Sdhaf1</i>	Succinate dehydrogenase complex assembly factor 1	3.247	102230433
<i>Rusc2</i>	RUN and SH3 domain containing 2	3.125	460168
<i>Syne2</i>	Synaptic nuclear envelope 2	2.924	102260348
<i>Col1a2</i>	Collagen, type I, alpha 2	2.801	380364
<i>Prpc</i>	Prolylcarboxypeptidase (angiotensinase C)	2.801	7050746
<i>Gpx2</i>	Glutathione peroxidase 2	2.717	6940619
<i>Gm3322</i>	Predicted gene 3322	2.710	6660128
<i>Timm9</i>	Translocase of inner mitochondrial membrane 9	2.611	1450019
<i>S100a9</i>	S100 calcium binding protein A9 (calgranulin B)	2.611	7050528
<i>BC003331</i>	cDNA sequence BC003331	2.525	1980164
<i>Mtap7d2</i>	MAP7 domain containing 2	2.469	106860494
<i>Rg9mtid1</i>	RNA (guanine-9-) methyltransferase domain containing 1	2.457	4480368
<i>Gch1</i>	GTP cyclohydrolase 1	2.445	670364
<i>Mmp3</i>	Matrix metalloproteinase 3	2.398	2650368
<i>Trem2</i>	Triggering receptor expressed on myeloid cells 2	2.398	6290358
<i>Trf</i>	Transferrin	2.381	1050193
<i>Adamts9</i>	A disintegrin-like and metalloproteinase (reprolysin type) with thrombospondin type 1 motif, 9	2.283	101580446
<i>Mmp3</i>	Matrix metalloproteinase 3	2.232	2970056
<i>H-T23</i>	Histocompatibility 2, T region locus 23	2.212	2850609
<i>Sorcs2</i>	Sortilin-related VPS10 domain containing receptor 2	2.208	100460068
<i>Wdr92</i>	WD repeat domain 92	2.203	5570097
<i>Fut10</i>	Fucosyltransferase 10	2.174	103120039
<i>Trim16</i>	Tripartite motif-containing 16	2.151	6510068
<i>Hps1</i>	Hermansky-Pudlak syndrome 1 homolog (human)	2.114	2510026
<i>Nipal1</i>	NIPA-like domain containing 1	2.079	6130315
<i>Aktip</i>	Thymoma viral proto-oncogene 1 interacting protein	2.066	6350575
<i>Txnl4a</i>	Thioredoxin-like 4A	2.062	4120286
<i>Gch1</i>	GTP cyclohydrolase 1	2.058	6550358
<i>Nupr1</i>	Nuclear protein 1	2.053	1990524
<i>Gstt2</i>	Glutathione S-transferase, theta 2	2.053	106650102
<i>Rad23a</i>	RAD23a homolog	2.045	670082
<i>Vps35</i>	Vacuolar protein sorting 35	2.045	7050687
<i>Cdkn2b</i>	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	2.033	6020040
<i>Hist1h4f</i>	Histone cluster 1, H4f	2.033	102450672
<i>Dbt</i>	Dihydrolipoamide branched chain transacylase E2	2.028	106180717

Klf5 in IS tissues of *Il15*^{+/+} compared with *Il15*^{-/-} mice on day 7.5 (Table 3), we did not detect a significant ($P > 0.05$) difference between these tissues using quantitative RT-PCR (qRT-PCR; Fig. 1C).

Expression of genes involved in angiogenesis

Previous work suggests that uNK cells express genes involved in angiogenesis and thus may play a role in endometrial angiogenesis during implantation (Lash *et al.* 2006, Li *et al.* 2008, Kalkunte *et al.* 2009). Indeed,

current dogma suggests that activated uNK cells are critical for endometrial angiogenesis that occurs in early implantation site development (Santoni *et al.* 2008, Petitbarat *et al.* 2010). Therefore, we expected abnormalities in uterine angiogenesis during decidualization in the IS tissue of the *Il15*^{-/-} mice due to the lack of uNK cells. Expression of disintegrin-like and metalloproteinase 9 (*Adamts9*) increases in the mouse uterus during implantation and is localized mainly to a subpopulation of vascular endothelial cells in the endometrium (Jungers *et al.* 2005). Since ADAMTS9 is an anti-angiogenic factor

Table 3 Genes whose steady-state mRNA levels were only significantly above background in IS tissue from day 7.5 *Il15*^{+/+} mice. Probe ID indicates Illumina Probe identification number.

Gene symbol	Name	Probe ID
2010011120Rik	RIKEN cDNA 2010011120 gene	102900270
6330403K07Rik	RIKEN cDNA 6330403K07 gene	2060504
<i>Abca5</i>	ATP-binding cassette, subfamily A (ABC1), member 5	1660110
<i>Avil</i>	Advillin	5690452
<i>CtsG</i>	Cathepsin G	1190711
<i>CtsW</i>	Cathepsin W	360368
<i>Fcho1</i>	FCH domain only 1	1240411
<i>Fgfr1op2</i>	FGFR1 oncogene partner 2	60538
<i>Fggy</i>	FGGY carbohydrate kinase domain containing	7100026
<i>Gm949</i>	Predicted gene 949	104480131
<i>Gzmd</i>	Granzyme D	4920368
<i>Gzme</i>	Granzyme E	1990112
<i>Gzme</i>	Granzyme E	5080292
<i>Gzmf</i>	Granzyme F	6200008
<i>Gzmg</i>	Granzyme G	1230341
<i>Klf5</i>	Kruppel-like factor 5	104150152
<i>Klrd1</i>	Killer cell lectin-like receptor, subfamily D, member 1	3190114
<i>Klrg1</i>	Killer cell lectin-like receptor subfamily G, member 1	2360133
<i>Ltb4r1</i>	Leukotriene B4 receptor 1	1770056
<i>Nkg7</i>	Natural killer cell group 7 sequence	1780390
<i>Nkg7</i>	Natural killer cell group 7 sequence	6370059
<i>Nmu</i>	Neuromedin U	6400025
<i>Prf1</i>	Perforin 1 (pore forming protein)	6660309
<i>Rasgef1c</i>	RasGEF domain family, member 1C	105420563
<i>Sh2d2a</i>	SH2 domain protein 2A	2970594
<i>Tcrb-V8.2</i>	T-cell receptor beta, variable 13-2	3610048
<i>Ttc27</i>	Tetratricopeptide repeat domain 27	1090280
<i>Xcl1</i>	Chemokine (C motif) ligand 1	3800504
<i>Zbtb32</i>	Zinc finger and BTB domain containing 32	5900397

(Koo *et al.* 2010), we verified that *Adamts9* mRNA levels were significantly ($P < 0.01$) greater in the IS tissues from *Il15*^{-/-} compared with those of *Il15*^{+/+} mice (Fig. 2A). On the other hand, given that uNK cells are believed to play a role in angiogenesis, it was surprising that the microarray analysis did not reveal differential expression of any other genes that may be involved in angiogenesis or that are expressed in endothelial cells. Therefore, we assessed the expression of several of these genes using qRT-PCR. *Vegfa*, *Vegfb*, *Vegfc*, vascular endothelial growth factor D/FOS-induced growth factor (*Figf*), as well as prostaglandin-endoperoxide synthase 2 (*Ptgs2*) and inhibin betaB (*Inhbb*) mRNA levels were not significantly ($P > 0.05$) different in the IS tissues from *Il15*^{+/+} compared with *Il15*^{-/-} mice on day 7.5 of pregnancy (Fig. 2A). To examine this in more detail, we measured mRNA levels of several other angiogenesis-related genes as well as endothelial cell markers in the IS tissue of *Il15*^{+/+} and *Il15*^{-/-} mice on days 6.5–10.5 of pregnancy. *Gja1*, *Angpt1*, *Angpt2*, and runt-related transcription factor 1 (*Runx1*) may play a role in uterine angiogenesis during implantation (Bany & Cross 2006, Hess *et al.* 2006, Ma & Zhu 2007, Laws *et al.* 2008, Dong & Chen 2009, Sur 2009). We verified that the expression of all four of these genes was not significantly ($P > 0.05$) different between the IS tissues from *Il15*^{+/+} and *Il15*^{-/-} mice on each day of pregnancy examined (Fig. 2B–E). Endothelial cells strongly express CD34

antigen (*Cd34*), platelet/endothelial cell adhesion molecule 1 (*Pecam1*), endothelial-specific receptor tyrosine kinase (*Tek*), FMS-like tyrosine kinase 1 (*Flt1*), kinase insert domain protein receptor (*Kdr*), and *Flt4* in the endometrium during decidualization (Wong *et al.* 1997, He *et al.* 1999, Halder *et al.* 2000, Matsumoto *et al.* 2002, Douglas *et al.* 2009). We verified no significant ($P > 0.05$) effect of genotype on the expression of all six of these genes between the IS tissues from *Il15*^{+/+} and *Il15*^{-/-} mice on each day of pregnancy examined except on day 9.5 for *Pecam1* and *Flt4* (Fig. 2F–K).

Table 4 Genes whose steady-state mRNA levels were only significantly above background in IS tissue from day 7.5 *Il15*^{-/-} mice. Probe ID indicates Illumina Probe identification number.

Gene symbol	Name	Probe ID
6720422M22Rik	RIKEN cDNA 6720422M22 gene	104590600
<i>Gfpt2</i>	Glutamine fructose-6-phosphate transaminase 2	102810242
<i>Ighg2c</i>	Immunoglobulin heavy constant gamma 2C	360113
<i>Prdx2</i>	Peroxisiredoxin 2	5340577
<i>Slpi</i>	Secretory leukocyte peptidase inhibitor	2120446
<i>Sspn</i>	Sarcospan	4670091
<i>Wdr46</i>	WD repeat domain 46	6020601

Figure 1 qRT-PCR assessment of the expression of genes involved in decidualization. (A) *Bmp2* mRNA and the mRNAs of *BMP2* downstream target genes plus progesterone receptor (*Pgr*) mRNA levels in IS tissue from day 7.5 pregnant *Il15*^{+/+} and *Il15*^{-/-} mice. Relative *Klf5* mRNA levels in non-implantation (NIS) and implantation (IS) segments of the uterus (B) or IS segments from *Il15*^{+/+} and *Il15*^{-/-} mice (C) on days 5.5–8.5 and days 6.5–8.5 of pregnancy respectively. Bars represent the mean $\pm$ S.E.M. ($n=4$) and statistically significant difference in mRNA levels is denoted by asterisks (* $P<0.05$).

Figure 2 qRT-PCR assessment of the expression of genes potentially involved in angiogenesis or vascular markers in the uterus. (A) Relative mRNA levels in IS tissue from day 7.5 pregnant *Il15*^{+/+} and *Il15*^{-/-} mice. Relative mRNA levels in IS tissues from *Il15*^{+/+} and *Il15*^{-/-} mice on days 6.5–10.5 of pregnancy for (B) *Gja1*, (C) *Angpt1*, (D) *Angpt2*, (E) *Runx1*, (F) *Cd34*, (G) *Pecam1*, (H) *Tek*, (I) *Flt1*, (J) *Kdr*, and (K) *Flt4*. Bars represent the mean $\pm$ S.E.M. ($n=4$) and statistically significant difference in mRNA levels is denoted by asterisks (* $P<0.05$, ** $P<0.01$).

Angiogenesis and vascular density changes during decidualization

Although it is clear that the characteristic of the spiral arteries by mid-pregnancy in mice depends on the presence of uNK cells, it is not solidly established that these cells play a role in angiogenesis of the uterus during decidualization. We speculated that vascular density and endothelial cell proliferation does not differ between the IS tissues of *Il15*^{+/+} and *Il15*^{-/-} mice given the results of the microarray and qRT-PCR analyses above. To assess this, we measured vascular density and endothelial cell proliferation in the IS tissues of *Il15*^{+/+} and *Il15*^{-/-} mice on days 6.5, 7.5, and 8.5 of pregnancy. As shown in Fig. 3A and B, we used CD34 staining to assess the localization and numbers of endothelial cells. We also used bromodeoxyuridine (BrdU) to identify those endothelial cells undergoing proliferation. We assessed three different regions including the antimesometrial (AM) region as well as the central (CM) plus lateral (LM) mesometrial regions (Fig. 3A and B). There was no effect ($P>0.05$) of genotype on endothelial cell density for each day examined in the CM (Fig. 3C), LM (Fig. 3D) or AM (Fig. 3E) areas of the endometrium. Similar findings were found for endothelial cell proliferation (Fig. 3F–H) except for the AM region in day 8.5 *Il15*^{-/-} mice where endothelial cells were not undergoing proliferation while a few were in the *Il15*^{+/+} mice.

Expression of genes involved in proteolysis

Many of the differentially expressed genes found in the microarray work are involved in proteolysis. So we verified the differential expression of several of these genes. Cathepsins are serine proteases, some of which have been shown to be expressed in uNK cells (Croy *et al.* 2010). The microarray results indicated a significant ($P<0.05$) decrease in cathepsin G (*Ctsg*) and cathepsin w (*Ctsw*) expression in the IS tissue of day 7.5 *Il15*^{-/-} mice. As determined by qRT-PCR, we found

Figure 3 Assessment of endothelial density and proliferation in the antimesometrial (AM), central mesometrial (CM), and lateral mesometrial (LM) regions of the endometrium of IS tissues of *Il15*^{+/+} and *Il15*^{-/-} mice during decidualization on days 6.5–9.5 of pregnancy. Photomicrographs of sections from IS tissue of (A) *Il15*^{+/+} and (B) *Il15*^{-/-} mice stained for CD34 (red fluorescence), BrdU (green fluorescence), and counterstained nuclei (DAPI, blue fluorescence). Endothelial cell density in (C) CM, (D) LM, and (E) AM areas on days 6.5–8.5 of pregnancy. Endothelial cell proliferation in (F) CM, (G) LM, and (H) AM areas on days 6.5–8.5 of pregnancy. Bars represent the mean $\pm$ S.E.M. ($n=4$).

the *Ctsg* mRNA levels were significantly ($P<0.05$) greater in IS compared with NIS tissues on days 7.5–8.5 of pregnancy from *Il15*^{+/+} mice (Fig. 4A). *Ctsg* mRNA levels were significantly ($P<0.05$) greater in the IS tissue of *Il15*^{+/+} mice compared with that of *Il15*^{-/-} mice on days 5.5, 6.5, 7.5, and 8.5 by ~ 40 -, 200-, 600- and 2000-fold respectively (Fig. 4B). Also known to be expressed in NK cells (Wex *et al.* 2001), *Ctsb* mRNA levels differed significantly ($P<0.05$) between NIS and IS tissues only on day 8.5 (Fig. 4C). However, *Ctsb* mRNA levels were significantly ($P<0.05$) greater in the IS tissue of *Il15*^{+/+} mice compared with that of *Il15*^{-/-} mice on days 5.5, 6.5, 7.5, and 8.5 by ~ 30 -, 15-, 19- and 34-fold respectively (Fig. 4D). Granzymes are another group of serine proteases, some of which have been shown to be expressed in uNK cells (Allen & Nilsen-Hamilton 1998).

The mRNA levels of several granzymes including granzyme D (*Gzmd*), E (*Gzme*), F (*Gzmf*), and G (*Gzmg*) were verified to be expressed at a significantly ($P<0.001$) greater level in day 7.5 IS tissues from *Il15*^{+/+} compared with that of *Il15*^{-/-} mice (Fig. 4E). Finally, expression of two protease genes, HtrA serine peptidase 1 (*Htra1*) and prolylcarboxypeptidase/angiotensinase C (*Prpcp*), was significantly ($P<0.05$) greater in the IS tissues from *Il15*^{+/+} and *Il15*^{-/-} mice respectively (Fig. 4E).

Expression of genes found previously to be expressed in uNK or NK cells

Killer cell lectin-like receptor subfamily D, member 1 (*Klr1d1*; Soderstrom *et al.* 1997, Verma *et al.* 1997, King *et al.* 2000, McGrath *et al.* 2009), *Klrg1* (Li *et al.* 2009), natural killer cell group 7 (*Nkg7*), perforin (*Prf1*) (Parr *et al.* 1990), secreted phosphoprotein 1 (*Spp1*; Herington & Bany 2007a), and tumor necrosis factor receptor superfamily member 9 (*Tnfrsf9*; Eckstrum & Bany 2011) genes have previously been shown to be expressed in uNK or NK cells. Since uNK cells are missing from the IS tissues of *Il15*^{-/-} mice (Barber & Pollard 2003, Croy *et al.* 2003b) and the microarray analysis identified the above-mentioned genes to be differentially expressed, we verified differential expression of these genes using qRT-PCR in the IS tissue from day 7.5 pregnant *Il15*^{-/-} compared with *Il15*^{+/+} mice (Fig. 4E).

Advillin expression

Since advillin (AVIL) is involved in cell morphogenesis (Shibata *et al.* 2004) and its expression was only seen in the IS tissue of *Il15*^{+/+} mice in the microarray analysis, we further characterized its expression in the uterus. *Avil* mRNA levels significantly ($P<0.05$) increased in the IS compared with NIS tissues of the uteri of *Il15*^{+/+} mice on days 6.5, 7.5, and 8.5 of pregnancy during decidualization (Fig. 5A). Previously, we found uNK cell numbers are significantly decreased in uteri undergoing bead-induced decidualization (BID) by days 7.5 and 8.5 (Herington & Bany 2007b), but in a recent microarray study, differences in *Avil* mRNA levels were not seen between the decidua and deciduoma on day 7.5 (McConaha *et al.* 2011). Therefore, we examined *Avil* mRNA levels in the IS tissue of pregnant mice and BID of pseudopregnant mice on days 4.5–8.5. There was a significant ($P<0.05$) decrease in *Avil* mRNA levels in BID compared with IS tissues only on day 8.5 (Fig. 5B). Next, we assessed the differential expression of *Avil* between the IS tissues from *Il15*^{+/+} and *Il15*^{-/-} mice on days 4.5–8.5 of pregnancy. As shown in Fig. 5C, *Avil* mRNA levels were significantly ($P<0.05$) greater in the IS tissues of *Il15*^{+/+} compared with *Il15*^{-/-} mice on days 6.5, 7.5, and 8.5 of pregnancy. Finally, to localize

Figure 4 qRT-PCR assessment of *Ctsg* and *Ctsw* expression in the pregnant mouse uterus. Relative *Ctsg* and *Ctsw* mRNA levels in non-implantation (NIS) and implantation (IS) segments of the uterus (A and B) or IS segments from *Il15^{+/+}* and *Il15^{-/-}* mice (C and D) on days 5.5–8.5 of pregnancy. (E) Relative mRNA levels of 12 genes in the IS tissues of day 7.5 pregnant *Il15^{+/+}* and *Il15^{-/-}* mice. Bars represent the mean $\pm$ S.E.M. ($n=4-6$) and statistically significant difference in mRNA levels is denoted by asterisks (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

where *Avil* is expressed, we carried out *in situ* hybridization on sections from day 7.5 (Fig. 5D and E) and day 8.5 (Fig. 5F and G) pregnant uteri from *Il15^{+/+}* mice. *Avil* mRNA was localized to a subgroup of cells in the mesometrial decidual region. To determine if this expression was localized to uNK cells, we used DBA lectin histochemistry coupled with *Avil* *in situ* hybridization. DBA lectin stains a subset of uNK cells in the mouse uterus during decidualization. As shown in Fig. 5H–J, the *Avil* mRNA was localized to a subset of DBA-positive uNK cells in the mesometrial decidua of day 7.5 mice. Not all DBA lectin-positive cells were *Avil*

mRNA positive, but all *Avil* mRNA-positive cells were DBA lectin positive. Finally, sections from *Il15^{-/-}* mice showed neither DBA-positive uNK cells nor *Avil* mRNA-positive cells in the mesometrial decidua (Fig. 5K).

Small proline-rich protein 2D expression

The expression of the large family of small proline-rich protein 2 (*Sprr2*) genes has been examined in the mouse endometrium and most of them are estrogen-regulated genes (Hong *et al.* 2004). Our microarray data suggested that *Sprr2d* mRNA levels are significantly ($P<0.05$) reduced in the IS tissue of day 7.5 pregnant uteri from *Il15^{-/-}* mice compared with those from *Il15^{+/+}* mice. To characterize the expression of the *Sprr2d* gene during decidualization, we examined expression in the uterus on days 4.5–8.5 of pregnancy. As shown in Fig. 6A, *Sprr2d* mRNA levels significantly ($P<0.05$) increased in IS compared with NIS tissues from the uteri of pregnant *Il15^{+/+}* mice on days 6.5, 7.5, and 8.5 of pregnancy by ~ 16 -, 29- and 214-fold respectively. Next, we examined the differences in *Sprr2d* mRNA levels in the IS tissues from *Il15^{+/+}* compared with *Il15^{-/-}* mice. As shown in Fig. 6B, mRNA levels were significantly greater in the IS tissues from *Il15^{+/+}* compared with *Il15^{-/-}* mice on days 7.5–8.5. We then carried out *in situ* hybridization to localize *Sprr2d* mRNA. As shown in Fig. 6C, *Sprr2d* mRNA was localized to the AM decidua in the IS tissue of *Il15^{+/+}* mice. Some mRNA was also localized to the same area in *Il15^{-/-}* mice, but to a lesser extent (Fig. 6C).

Degenerative spermatocyte homolog 2 expression

Our microarray data suggested that degenerative spermatocyte homolog 2 (*Dege2*) mRNA levels are significantly reduced in the IS tissue of day 7.5 pregnant uteri from *Il15^{-/-}* mice compared with those from *Il15^{+/+}* mice. Since the levels of one of the products of DEGS2 action, ceramide, increases in the uterus during implantation and may play a role in decidualization (Kaneko-Tarui *et al.* 2007), we characterized the expression of *Dege2* in the uterus during implantation. As shown in Fig. 6D, *Dege2* mRNA levels were significantly ($P<0.05$) greater in NIS compared with IS tissue from the uteri of pregnant *Il15^{+/+}* mice on days 5.5 and 6.5 of pregnancy. Next, we examined the differences in *Dege2* mRNA levels in IS tissues from *Il15^{+/+}* compared with *Il15^{-/-}* mice. As shown in Fig. 6E, mRNA levels were significantly greater in the IS tissues from *Il15^{+/+}* compared with *Il15^{-/-}* mice on days 7.5 and 8.5 of pregnancy. Finally, we carried out *in situ* hybridization to localize *Dege2* expression in the uteri on day 7.5 of pregnancy. As shown in Fig. 6F, *Dege2* mRNA was found in mesometrial decidual cells just adjacent to the conceptus in IS tissue from *Il15^{+/+}* mice, but not in that of *Il15^{-/-}* mice.

Figure 5 *Avil* expression in the mouse uterus during decidualization and its localization to uNK cells using qRT-PCR and *in situ* hybridization. (A) Relative *Avil* mRNA levels between NIS and IS tissues of pregnant mouse uterus on days 4.5–8.5 of pregnancy. (B) Relative *Avil* mRNA levels between IS tissue and BID tissue of the mouse uterus on days 4.5–8.5 of pregnancy and pseudopregnancy respectively. (C) Relative *Avil* mRNA levels between IS tissues from *Il15*^{+/+} and *Il15*^{-/-} mice on days 4.5–8.5 of pregnancy. Bars represent the mean $\pm$ S.E.M. ($n=3-4$) and * denotes a statistically significant ($P<0.05$) difference between bars on a given day. Localization of *Avil* mRNA in IS tissue of *Il15*^{+/+} mice on (D and E) day 7.5 and (F and G) day 8.5 of pregnancy. Localization of *Avil* mRNA and DBA lectin-positive uNK cells in IS tissue of (H–J) *Il15*^{+/+} and (K) *Il15*^{-/-} mice on day 7.5 of pregnancy. Scale bars are in microns.

Cytokine-induced apoptosis inhibitor 1 expression

Our microarray data suggested that cytokine-induced apoptosis inhibitor 1 (*Ciabin1*) mRNA levels are significantly reduced in the IS tissue of day 7.5 pregnant uteri from *Il15*^{-/-} mice compared with those from *Il15*^{+/+} mice. Since RAS plays a role in decidualization (Cho *et al.* 2011) and *Ciabin1* operates downstream of the receptor tyrosine kinase-Ras signaling pathway (Hao *et al.* 2006), we characterized *Ciabin1* mRNA levels in the mouse uterus during decidualization. As shown in Fig. 6G, *Ciabin1* mRNA levels increased in IS compared with NIS tissues from the uteri of pregnant *Il15*^{+/+} mice on days 6.5–8.5 of pregnancy. Next, we examined the differences in *Ciabin1* mRNA levels in the IS tissues from *Il15*^{+/+} compared with *Il15*^{-/-} mice. As shown in Fig. 6H, mRNA levels were significantly greater in the IS tissues from *Il15*^{+/+} compared with *Il15*^{-/-} mice on days 5.5–8.5 of pregnancy. Finally, we carried out *in situ* hybridization to localize *Ciabin1* expression in the uteri on day 7.5 of pregnancy. As shown in Fig. 6I, *Ciabin1* mRNA was found in mesometrial decidual cells just adjacent to the ectoplacental cone and in the AM decidua adjacent to the myometrium in IS tissue from *Il15*^{+/+} mice. A similar signal for *Ciabin1* mRNA in tissues from *Il15*^{-/-} mice was only seen in the AM stromal cells in IS tissues.

Annexin A8 expression

Annexin A8 (*Anxa8*) encodes a protein that plays a role in late endosome organization and function (Goebeler *et al.* 2008). Our microarray data suggested that *Anxa8* mRNA levels are significantly reduced in the IS tissue of day 7.5 pregnant uteri from *Il15*^{-/-} mice compared with those from *Il15*^{+/+} mice. To characterize the expression of this gene during decidualization, we examined its expression in the uterus on days 3.5–8.5 of pregnancy. As shown in Fig. 7A, *Anxa8* mRNA levels increased in IS compared with NIS tissues from the uteri of pregnant *Il15*^{+/+} mice on days 5.5–8.5 of pregnancy. Next, we examined the differences in *Anxa8* mRNA levels in the IS tissues from *Il15*^{+/+} compared with those from *Il15*^{-/-} mice. As shown in Fig. 7B, mRNA levels were significantly greater in IS tissues from *Il15*^{+/+} compared with those from *Il15*^{-/-} mice on day 7.5.

RAP1 GTPase-activating protein expression

Although little is known about the function of RAP1 GTPase-activating protein (*Rap1gap*) expression in the uterus during implantation, we characterized its expression in the uterus since the microarray data suggested that its mRNA levels are significantly reduced

Figure 6 (A–C) *Spr2d*, (D–F) *Degs2*, and (G–I) *Ciapin1* expression and localization in the mouse uterus during decidualization using qRT-PCR and *in situ* hybridization respectively. (A, D and G) Relative mRNA levels between NIS and IS tissues of pregnant mouse uterus on days 4.5–8.5 of pregnancy. (B, E and H) Relative mRNA levels between IS tissues from *Il15*^{+/+} and *Il15*^{-/-} mice on days 4.5–8.5 of pregnancy. Bars represent the mean $\pm$ S.E.M. ($n=3-4$) and * denotes a statistically significant ($P<0.05$) difference between bars on a given day. (C, F and I) Localization of mRNA in IS tissue of *Il15*^{+/+} (left panel) and *Il15*^{-/-} (right panel) mice on day 7.5 of pregnancy.

in the IS tissue of day 7.5 pregnant uteri from *Il15*^{-/-} mice compared with those from *Il15*^{+/+} mice. As shown in Fig. 7C, relative *Rap1gap* mRNA levels significantly ($P<0.05$) increased in IS compared with NIS tissues on days 6.5–9.5 of pregnancy. As shown in Fig. 7D, mRNA levels were also significantly ($P<0.05$) greater in IS tissues from *Il15*^{+/+} compared with those from *Il15*^{-/-} mice on these days.

Zinc finger and BTB domain containing 32 expression

Zinc finger and BTB domain containing 32 (*Zbtb32*) is a transcriptional repressor that negatively regulates T-cell activation (Kang *et al.* 2005). Although little is known

about the function of *Zbtb32* expression in the uterus during implantation, we characterized its expression in the uterus since the microarray data suggested that its mRNA levels are significantly reduced in the IS tissue of day 7.5 pregnant uteri from *Il15*^{-/-} mice compared with those from *Il15*^{+/+} mice. Relative mRNA levels of *Zbtb32* were found to be significantly ($P<0.05$) increased in IS compared with NIS tissues of the pregnant uterus on days 6.5, 7.5, and 8.5 of pregnancy by an average of 7-, 15-, and 35-fold respectively (Fig. 7E). As shown in Fig. 7F, mRNA levels were also significantly ($P<0.05$) greater in the IS tissues from *Il15*^{+/+} compared with those from *Il15*^{-/-} mice on days 6.5–8.5.

Figure 7 (A and B) *Anxa8*, (C and D) *Rap1gap*, and (E and F) *Zbtb32* expression and localization in the mouse uterus during decidualization using qRT-PCR and *in situ* hybridization respectively. (A, C and E) Relative mRNA levels between NIS and IS tissues of pregnant mouse uterus on days 4.5–8.5 of pregnancy. (B, D and F) Relative mRNA levels for IS tissue from *Il15*^{+/+} and *Il15*^{-/-} mice on days 4.5–8.5 of pregnancy. Bars represent the mean $\pm$ S.E.M. ($n=3-4$) and * denotes a statistically significant ($P<0.05$) difference between bars on a given day.

Further independent verification of microarray data

We verified the differential expression of several additional genes using qRT-PCR. As shown in Fig. 8, we attempted to verify differential levels of mRNAs between the IS tissues from *Il15*^{+/+} and *Il15*^{-/-} mice for an additional 18 genes. As shown in Fig. 8A, we verified a significantly greater mRNA level in the IS tissue from *Il15*^{+/+} mice for coronin actin binding protein 1A (*Coro1a*, $P<0.05$), diamine oxidase-like protein 2 (*Doxl2*, $P<0.05$), glycerophosphodiester phosphodiesterase domain containing 3 (*Gdpd3*, $P<0.005$), NECAP endocytosis associated 2 (*Necap2*, $P<0.001$), neuromedin U (*Nmu*, $P<0.05$), platelet-derived growth factor receptor-like (*Pdgfrl*, $P<0.001$), phospholipase C gamma 1 (*Plcg1*, $P<0.001$), proline-serine-threonine phosphatase-interacting protein 1 (*Pstpip1*, $P<0.001$), selectin platelet ligand (*Selplg*, $P<0.001$), serine peptidase inhibitor Kazal type 2 (*Spink2*, $P<0.05$), chemokine (C motif) ligand 1 (*Xcl1*, $P<0.001$), and RIKEN cDNA

6330403K07 (*6330403KO7Rik*, $P<0.05$). As shown in Fig. 8B, we verified a significantly greater mRNA level in the IS tissue from *Il15*^{-/-} compared with that from *Il15*^{+/+} mice for GTP cyclohydrolase 1 (*Gch1*, $P<0.05$), glutathione peroxidase 2 (*Gpx2*, $P<0.001$), MAP7 domain containing 2 (*Mtap7d2*, $P<0.001$), regulator of G-protein signaling 5 (*Rgs5*, $P<0.05$), calcium binding protein A9 (*S100a9*, $P<0.05$), short coiled-coil protein (*Scoc*, $P<0.005$), *Spink3* ($P<0.05$), and RIKEN cDNA 6720422M22 (*6720422M22Rik*, $P<0.01$).

Discussion

The developing decidual tissue provides a critical nutritive environment for the conceptus before the establishment of the functional placenta. The major immune cells present in the uterus during decidualization are uNK cells and they are present in the mesometrial decidua during decidualization (Zhang *et al.* 2009). However, the role of the immune system in the process of decidualization is currently not well understood. The availability of several genetic models, where uNK cells are absent during pregnancy, makes such an assessment possible. *Il15*-deficient mice lack uNK cells in the uterus during implantation and this genetic model had been used previously to study uNK cell function in the uterus during implantation in mice (Ashkar *et al.* 2003, Barber & Pollard 2003, Croy *et al.* 2003a). This work clearly supports a role for uNK cells in ‘maintenance of decidual integrity’ based on the observation that the decidual basalis after mid-pregnancy is acellular in uNK-deficient mice (Ashkar *et al.* 2003). Notably, the use of this and other similar models of uNK cell deficiency has not revealed if uNK cells play a role in decidualization itself, which occurs in the uterus during the 4–5 days of pregnancy leading up to mid-pregnancy. Thus, the present study was initially undertaken to determine the differences in uterine gene expression in IS tissues between *Il15*^{+/+} and *Il15*^{-/-} mice during decidualization using whole-genome Illumina BeadArray analysis. The microarray results suggested that 300 genes were differentially expressed and the differential mRNA levels of approximately one-sixth of these were verified independently using qRT-PCR. Interestingly, the present study did not reveal any differential expression of key genes known to play roles in endometrial stromal cell decidualization or of genes that are decidual cell markers. Therefore, the results of this study support the hypothesis that uNK cells do not play a critical role in decidualization and the loss of decidual integrity seen after mid-pregnancy in *Il15*^{-/-} mice is not due to a defect in decidual cell differentiation.

Decidualization involves a rapid growth of the endometrial tissue mass and is accompanied by angiogenesis. Several genetic models, including *Il15*^{-/-}, have established a critical role for uNK cells in the spiral arteriole modification seen after mid-pregnancy.

Figure 8 Independent verification of the differential expression of 20 additional genes using qRT-PCR. Relative mRNA levels of 12 and 8 genes found to be expressed at higher levels in IS tissue of (A) *IL15*^{+/+} and (B) *IL15*^{-/-} mice by microarray analysis. Bars represent the mean $\pm$ S.E.M. ($n=3-6$) and statistically significant difference in mRNA levels is denoted by asterisks (* $P<0.05$, ** $P<0.01$, *** $P<0.005$, and **** $P<0.001$).

Further, it has been speculated that uNK cells may play a critical role in endometrial angiogenesis during decidualization or implantation in rodents and humans due to the observations that they are a source of angiogenic factors (Lash *et al.* 2010, Zhang *et al.* 2011). The expression of several genes such as *Vegfa* (Halder *et al.* 2000), *Ptgs2* (Lim *et al.* 1997, Matsumoto *et al.* 2002), *Gja1* (Laws *et al.* 2008), *Angpt2* (Matsumoto *et al.* 2002, Hess *et al.* 2006), *Tie2* (Matsumoto *et al.* 2002), *Flt1*, and *Kdr* (Chakraborty *et al.* 1995, Douglas *et al.* 2009) is believed to play a role in angiogenesis in the endometrium during decidualization. Further, several endothelial vascular endothelial cell markers such as *Flt4* (Douglas *et al.* 2009), *Cd34* (Morison *et al.* 2007), *Pecam1* (Ma *et al.* 1997), *Adamts9* (Jungers *et al.* 2005), and *Inhbb* (McConaha *et al.* 2011) have been shown to be expressed in the endometrial endothelial cells during decidualization. Notably, with the exception of *Adamts9* which is an anti-angiogenic factor, none of the other genes were differentially expressed in IS tissues between *IL15*^{+/+} and *IL15*^{-/-} mice. This included genes that encode angiogenic factors such as *Vegfa* and *Vegfc* which have previously been shown to be expressed by mouse and human uNK cells respectively (Wang *et al.* 2000, 2003, Lash *et al.* 2006). Therefore, uNK cells appear not to be the major source of *Vegfa* expression in the mouse uterus during decidualization. Finally, vascular morphometric analysis in the present study revealed very little difference in the endothelial cell

densities and endothelial cell proliferation indices between IS tissues from *IL15*^{+/+} and *IL15*^{-/-} mice during decidualization. Taken together, the data of this study suggest that uNK cells do not play a major role in angiogenesis or neovascularization in the endometrium during decidualization. Therefore, the lack of decidual integrity in genetic models that lack uNK cells is likely not due to decreased angiogenesis and impaired blood flow to the developing decidua during decidualization.

The cathepsin family of genes encodes either serine- or cysteine-proteases found in lysosomes within cells. Previous work has shown that the cathepsin D gene (*Ctsd*) is expressed in mature uNK cells in the mouse uterus by mid-pregnancy (Croy *et al.* 2010). However, whether uNK cells are a major source of other cathepsins remains to be determined. Therefore, it was interesting that the present study did not reveal a significant decrease in *Ctsd* expression in the IS tissue of *IL15*^{-/-} mice but did for *Ctsg* and *Ctsw*. CTSG is found in neutrophil lysozymes and upon secretion it can enhance the activity of interleukin-8, decrease the bioavailability of interleukin-6, activate cytokines (interleukin-1 beta, tumor necrosis factor alpha), and activate certain receptors (epidermal growth factor, proteinase activated receptors) (Conus & Simon 2010). Although it still needs to be proven, we speculate that the reduction of *Ctsg* expression found in the IS tissue of *IL15*^{-/-} mice may be due to a decrease in neutrophil numbers. *Ctsw* is exclusively expressed in NK cells as well as CD8-positive T cells but is not involved in cytotoxicity or interferon gamma production (Stoeckle *et al.* 2009). Unlike *Ctsg*, where there is an increased expression in IS tissues during decidualization, *Ctsw* expression in pregnancy appears to be complex because expression levels are similar in NIS and IS tissues until day 8.5. The present study shows that *Ctsg* expression and *Ctsw* expression in the mouse uterus during decidualization are both dependent on IL15 and further work to localize expression and determine their function in the uterus during this process is warranted.

Several genes known to be expressed by uNK cells of the uterus during the establishment of pregnancy or in NK cells in general were expected to be expressed at significantly lower levels in IS tissue from *IL15*^{-/-} mice compared with those of *IL15*^{+/+} mice. This included some granzymes (Allen & Nilsen-Hamilton 1998) and killer cell lectin-like receptor (Soderstrom *et al.* 1997, Verma *et al.* 1997, King *et al.* 2000, McGrath *et al.* 2009) genes along with *Ctsd* (Croy *et al.* 2010), *Prf1* (Parr *et al.* 1990), *Spp1* (Herington & Bany 2007a), and *Tnfrsf9* (Eckstrum & Bany 2011). Such a result was expected as uNK cells would be a major source of this expression in the uterus during decidualization. Indeed, many of these genes (but not all) were actually shown to be differentially expressed. However, it is notable that of the four genes examined further using *in situ* hybridization, only *Avil* was shown to be expressed in uNK cells.

Since expression of *Spr2d*, *Degs2*, and *Ciabin1* was not found in uNK cells, the control of their expression by IL15 may be more complex. Whether or not all of the other genes found to be differentially expressed at higher levels in IS tissues from *Il15*^{+/+} compared with *Il15*^{-/-} mice are expressed in uNK cells will require further work on each gene.

AVIL is an intracellular protein that belongs to the gelsolin superfamily of actin regulatory proteins (Silacci *et al.* 2004). The present study demonstrated that the expression of *Avil* in the endometrium during decidualization is located to uNK cells. Although the precise function of AVIL in uNK cell biology is not understood at present, it is known to interact with a protein called scavenger receptor member 1 (SCARF1; Shibata *et al.* 2004). AVIL is believed to play a role in cell morphogenesis through its interaction with SCARF1 (Shibata *et al.* 2004). Since uNK cells undergo a great deal of changes in cell size and shape as they mature in the endometrium during decidualization (He *et al.* 1999), we speculate that AVIL may be playing a role in uNK maturation. However, SCARF1 is also responsible for binding to and internalization of extracellular heat shock proteins, which may be important in cell signaling and immunity (Calderwood *et al.* 2007a, 2007b). Thus, the precise function of AVIL in the endometrium during decidualization remains to be determined.

Ceramide levels increase in the uterus during decidualization where it may play a role in apoptosis. Apoptosis occurs in the epithelia and stromal compartments of the endometrium during implantation, but little is known on how this is regulated (Pampfer & Donnay 1999, Joswig *et al.* 2003). Ceramide levels are increased by a diverse number of stress stimuli and ceramide causes suppression in cell growth and promotion of apoptosis (Ruvolo 2003). It appears that the balance of ceramide production and breakdown pathways seems to favor an increased accumulation in the uterus since ceramide levels in the mouse uterus increase during decidualization (Kaneko-Tarui *et al.* 2007). One major pathway of ceramide production is via serine where the last step involves the dihydroceramide $\Delta 4$ -desaturase and C-4 hydroxylase activities of DEGS2, which produces ceramide from dihydroceramide (Enomoto *et al.* 2006). The present study shows that *Degs2* mRNA is localized to the mesometrial decidua and its level is significantly reduced in the IS site of *Il15*^{-/-} mice compared with that of *Il15*^{+/+} mice. Therefore, it is possible that ceramide-mediated apoptosis of the mesometrial decidua may be occurring to make place for the developing placenta. Notably, we also characterized the expression of another apoptosis-related gene in this study, *Ciabin1*. *Ciabin1* encodes a protein originally called anamorsin that also plays roles in apoptosis as a downstream effector of RAS-signaling in the uterus (Hao *et al.* 2006). *Ciabin1* was also expressed in a region similar to that of *Degs2* in the mesometrial

decidua. Its expression levels dramatically increased in the IS tissue during implantation. In addition, expression of *Ciabin1* in the IS tissue of *Il15*^{-/-} mice was dramatically reduced during decidualization compared with that of *Il15*^{+/+} mice. Therefore, it appears that the expression of both *Degs2* and *Ciabin1* in the uterus is dependent on IL15. Whether or not this involves the loss of uNK cells or occurs through another mechanism remains to be studied further, as do the potential roles of these genes in apoptosis in the mesometrial decidua during implantation.

In conclusion, this study provides the identity of many genes whose expression in the uterus during decidualization is altered between *Il15*^{+/+} and *Il15*^{-/-} mice. However, only speculation on the potential function of many of these genes in the uterus during decidualization may be derived from the literature because the work was conducted on non-uterine tissues or cell types. For example, the present study shows the transcriptional repressor *Zbtb32* is expressed at high levels in the IS tissues of the uterus during decidualization in *Il15*^{+/+} mice and this expression is mainly lost in *Il15*^{-/-} mice. Since it has been shown in other tissues/cells that ZBTB32 acts as a repressor of the activity of GATA transcription factors, one can speculate that it may play a similar role in the uterus. However, currently, very little is known about uterine GATA transcription factor gene expression and function in the uterine compartment during decidualization. Another example of a gene found to be differentially expressed in this study was *Rap1gap*. RAP1GAP is known to activate mitogen-activated or extracellular (MAP/ERK) kinase signaling cascades in other tissues/cell types by increasing the rate of GTP hydrolysis, which in turn causes the inactivation of RAP GTPases (Ika *et al.* 2009, Tsygankova *et al.* 2010). However, to the best of our knowledge, nothing is known about the function of *Rap1gap* expression in the uterus during decidualization. In summary, the functions of many of the other genes found to be differentially expressed in this study still remain to be determined and can only be speculated at this time. However, this study should form the basis of future research on these genes.

Materials and Methods

Mice and sample collection

All procedures involving mice were approved by the Southern Illinois University Institutional Animal Care and Use Committee. They were maintained under controlled light conditions (lights on from 0700 to 2100 h) and were allowed free access to food and water. CD1 mice with a targeted deletion of the *Il15* gene (*Il15*^{-/-}) as well as wild-type (*Il15*^{+/+}) and *Il15*^{-/-} virgin females (8–12 weeks) were mated with *Il15*^{-/-} and wild-type male CD1 mice respectively. Wild-type CD1 mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and generation of the *Il15*^{-/-} mice on a CD1 background was previously described (Eckstrum & Bany 2011). The morning

a vaginal plug was detected was considered to be day 0.5 of pregnancy. These pregnant mice, all of which carried heterozygous embryos/conceptuses, were killed at 0900 h on days 4.5–9.5 of pregnancy. The bead-induced decidualization model was used exactly as described previously (McConaha *et al.* 2011).

RNA isolation

For day 4.5–8.5 uterine horns, NIS IS segments were separated. For IS segments, tissues of the conceptus (embryo and trophoblast cells) were carefully dissected out and discarded. All the remaining tissues were then pooled and considered IS segment tissue. Tissues were stored in RNA Later (Life Technologies, Grand Island, NY, USA). Total RNA was isolated using Trizol reagent as recommended by the manufacturer (Invitrogen). The RNA was then subjected to DNase digestion using Turbo DNase as recommended by the manufacturer (Ambion), followed by re-extraction with Trizol.

Microarray analysis

RNA samples isolated from the uterine tissue of day 7.5 IS segments in *Il15*^{+/+} and *Il15*^{-/-} mice were used for the microarray analysis ($n=3$). The RNA quality was verified using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) before microarray analysis as previously described (McConaha *et al.* 2011). Briefly, total RNA isolated from the tissue for each experiment was used to create biotin-labeled cRNA target samples ($n=3$) using the Illumina RNA amplification kit (Applied Biosystems, Carlesbad, CA, USA). The cRNA target preparations were then hybridized to the arrays for 16–20 h at 55 °C, and the bound biotin-labeled targets were then detected using streptavidin-Cy3 and an Illumina Bead Array Reader with BeadStudio software (Illumina, Inc. San Diego, CA, USA). Probe data were filtered to include only those with detection scores >0.98 ($P<0.02$) for all samples. The data were then exported to Chipster for further analysis (Kallio *et al.* 2011). The quantile normalization (Bolstad *et al.* 2003) tool of Chipster, which uses the limma R package, was used to normalize the data. Significance of differential expression was determined with the Chipster tool that uses the Bioconductor package limma Empirical Bayes method (Smyth 2004). The default Benjamini–Hochberg method was used to control the false discovery rate. Differences were considered significant if the adjusted P values were <0.05 . Finally, the raw data were filtered to find probe signals which were significantly above background in all three samples from all three *Il15*^{+/+} or *Il15*^{-/-} mice. This was defined as a detection score >0.98 , a signal level >150 plus an average of fourfold or greater signal above background. The lists of probes and relative signal levels were combined and annotated using the Bioconductor annaffy package of Chipster. Probes were removed from further analysis and from the lists of differentially expressed genes if they did not map to a gene or were mapped to either more than one gene or a pseudogene. Finally, the hypogeometric test for GO tool of Chipster was used to determine over- and under-represented GO terms of the list of differentially expressed genes using a cutoff of $P<0.01$ for statistical significance.

qRT-PCR analysis

qRT-PCR was carried out as described previously (McConaha *et al.* 2011) using the oligonucleotide primers (IDT Technologies, Coralville, IA, USA) shown in Supplementary Table 3, see section on supplementary data given at the end of this article. Briefly, High Capacity RNA-to-cDNA kits (Applied Biosystems) were used to generate the cDNA. PCR was performed using 2x iQ SYBR Green Supermix and a CFX real-time PCR machine (Bio-Rad). PCR conditions were 3 min at 94 °C followed by 40 cycles of 94 °C, 62–65 °C for 20 s, and 72 °C for 1 min for melting, annealing, and extension steps respectively. The PCR primers were used at a final concentration of 200 nM. Cycle thresholds (C_t) were determined using the CFX software, and relative mRNA levels were determined as described previously after normalization to 18S rRNA levels (McConaha *et al.* 2011). The efficiencies of the PCR were linear between the mRNAs and 18S rRNA and were $>85\%$. The effects of day of pregnancy and site of sampling on mean relative steady-state mRNA levels between NIS and IS tissues were determined statistically using two-way repeated measures ANOVA. Likewise, the effect of day of pregnancy and mouse genotype on mean relative steady-state mRNA levels in IS tissue from *Il15*^{+/+} and *Il15*^{-/-} mice was determined statistically using two-way repeated measures ANOVA. ANOVA was followed by the use of Duncan multiple range tests to determine differences between the means when warranted. Data collected on a single day of pregnancy were analyzed using t -tests to determine the effect of genotype on mRNA levels in IS tissues. All statistical analyses were conducted using SigmaPlot software (Systat Software, Inc., San Jose, CA, USA).

In situ hybridization

To collect tissue for *in situ* hybridization, mice were anesthetized, perfused with PBS, and then fixed with paraformaldehyde as described previously (McConaha *et al.* 2011). Processing of tissue into paraffin was done using routine methods. Five micrometer sections were mounted onto HistoBond glass slides (Statlab, McKinney, TX, USA). Templates for sense and antisense riboprobe synthesis were prepared by PCR and cloned into pGEM-T Easy as described previously (McConaha *et al.* 2011). PCR primers used are shown in Supplementary Table 3. After sequencing the cDNA clones (UIUC DNA Sequencing Lab), the plasmids were used to generate transcription templates by PCR as described previously (McConaha *et al.* 2011). After purification of the DNA using a QIAquick PCR purification kit (Qiagen), the template was used to generate digoxigenin (DIG)-labeled riboprobes using MAXIscript T7 and SP6 kits (Applied Biosystems) along with digoxigenin-11-UTP (Roche). After riboprobe purification using MEGAclear kits (Applied Biosystems), *in situ* hybridization was performed using antisense DIG-labeled riboprobes exactly as described in detail elsewhere (Simmons *et al.* 2007). Both antisense and control sense DIG-labeled riboprobes were used for *in situ* hybridization. Use of sense probes for all target genes studied in the *in situ* hybridization work of this study did not result in any positive

hybridization signals (see [Supplementary Figure 1](#), see section on [supplementary data](#) given at the end of this article). In some cases, after completion of the *in situ* staining, the slides were processed for DBA lectin histochemistry exactly as described elsewhere (Eckstrum & Bany 2011).

Immunohistochemistry and vascular morphometry

To visualize those cells undergoing proliferation, mice were injected with BrdU 4 h before tissue collection as previously described (Herington & Bany 2007b). Uterine sections were de-waxed and hydrated using routine techniques and then exposed to 0.2% trypsin in PBS at 37 °C for 10 min for antigen retrieval. After washing the sections in PBS, they were incubated with 1.5 M HCl for 15 min and then washed with water. To neutralize the sections, they were covered with borate buffer (0.1 M, pH 8.5) for 10 min and then washed in PBS. Subsequent incubations were carried out using antibody amplifier trays from ProHisto (Columbia, SC, USA) and a horizontal shaker. Sections were next incubated in blocking buffer containing 2% donkey serum in PBS with 0.05% tween (PBST) for 60 min. Sections were then incubated with 0.3 µg/ml sheep anti-BrdU (Bioscience International, Saco, ME, USA) and rat anti-mouse CD34 IgG (0.1 µg/ml) (Cedarlane Laboratories, Hornby, ON, Canada) for 1 h. After washing in PBST, the sections were incubated with 0.5 µg/ml biotinylated donkey anti-rat IgG and 1 µg/ml Dye-light 488 donkey anti-sheep IgG in blocking medium (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 60 min. After washing the sections in PBST, they were incubated with 0.5 µg/ml Dye-light 549-conjugated Streptavidin (Jackson ImmunoResearch Laboratories) in PBS for 30 min. After washing with PBST, the slides were incubated with 4',6-diamidino-2-phenylindole (DAPI) in PBS for 10 min and then washed in PBS. Finally, coverslips were placed over the sections using Fluoromount G mountant (Southern Biotech, Birmingham, AL, USA).

Slides were examined using a Leica CTR5000 fluorescence microscope equipped with a Retiga 2000JR QImaging Camera and using Qcapture Pro software (QImaging, Burnaby, BC, Canada). Endothelial cells were counted as CD34 cells lining the lumens of blood vessels, and BrdU-positive cells were counted as proliferating cells. Photomicrographs were taken with a 20× objective in CM plus LM and AM regions of the endometrium, and total BrdU-positive and -negative endothelial cells were manually counted in enlarged prints of the photomicrographs from AM and LM and CM uterine regions (from four independent samples). Endothelial cell density was calculated as the number of endothelial cells normalized to area, while proliferation index was calculated as the percent of BrdU-positive endothelial cells or vessels. A minimum of 200 endothelial cells per area were counted in each independent sample. Two-way ANOVA was performed to detect overall differences in the uNK cell subtype densities for each individual day and sub-region using SigmaPlot software (Systat Software, Inc., Chicago, IL, USA). This was followed by the use of Duncan multiple range tests to determine differences between the means.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0325>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by an NIH – Eunice Kennedy Shriver National Institute of Child Health and Human Development (HD049010) grant (to B M Bany). Support from Southern Illinois University was received in the form of Undergraduate Research Assistantships (to K S Eckstrum and C A Scott) and an Undergraduate REACH Award (to K S Eckstrum).

Acknowledgements

The authors thank Jen Herington and Melinda McConaha for technical assistance with some of the real-time PCR assays and cDNA clone preparation respectively.

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Received 31 August 2011

First decision 26 October 2011

Revised manuscript received 23 November 2011

Accepted 20 December 2011