A study on regional differences in decidualization of the mouse uterus

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Abstract

Although regional differences in mouse decidualization have been recognized for decades, the molecular mechanisms remain understudied. In the present study, by using RNA-seq, we compared transcriptomic differences between the anti-mesometrial (AM) region and the mesometrial (M) region of mouse uterus on day 8 of pregnancy. A total of 1423 differentially expressed genes were identified, of which 811 genes were upregulated and 612 genes were downregulated in the AM region compared to those in the M region. Gene ontology analysis showed that upregulated genes were generally involved in cell metabolism and differentiation, whereas downregulated genes were associated with lymphocyte themes and immune response. Through network analysis, we identified a total of 6 hub genes. These hub genes are likely more important than other genes due to their key positions in the network. We also examined the promoter regions of differentially expressed genes for the enrichment of transcription factor-binding sites. In the end, we demonstrated that a similar regional gene expression pattern can be observed in the artificial decidualization model. Our study contributes to an increase in the knowledge on the molecular mechanisms underlying regional decidualization in mice.

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Introduction

One of the essential steps for the establishment and maintenance of human pregnancy is the decidualization of endometrial stromal cells (Wang & Dey 2006). During decidualization, stromal cells undergo proliferation and subsequent differentiation into large epithelioid cells characterized by the secretion of decidual prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP1) (Telgmann & Gellersen 1998). Decidualization has various functional roles, such as the regulation of embryo implantation, modulation of maternal immune response to embryonic antigens and control of placentation (Blois *et al.* 2011, Barrientos *et al.* 2014). Defects in decidualization during early pregnancy may lead to severe pregnancy complications, such as pregnancy loss, intrauterine growth restriction and pre-eclampsia (Gellersen & Brosens 2014).

The mouse is widely used as an animal model for the study of human pregnancy. Human decidualization initiates spontaneously in the secretory phase of menstrual cycle in response to steroid hormones. Mouse decidualization is slightly different in that it is an embryo-dependent process (Ramathal *et al.* 2010). Mouse decidualization occurs in a temporal and regional fashion (Dey *et al.* 2004). Shortly after embryo

implantation, stromal cells adjacent to the implanting embryo in the anti-mesometrial (AM) region of the uterus show the first sign of decidualization reaction, which form the primary decidual zone (PDZ). Decidualization then spreads rapidly throughout the AM region, forming the secondary decidual zone (SDZ). Meanwhile, stromal cells in the mesometrial (M) region of uterus begin to undergo decidualization to form the mesometrial decidual area. The decidualized cells in the AM region and the M region are morphologically different. The AM region is a compact tissue with a portion of polyploid cells, whereas the M region is not a compact tissue and polyploidy is absent. From a functional perspective, decidualization in the AM region is involved in the accommodation of embryo implantation and decidualization in the M region contributes to the preparation of placentation.

However, the molecular information about regional differences in decidualization currently remains unavailable. In the present study, we used RNA-seq approach to investigate the gene expression profiles for the AM region and the M region of mouse uterus on day 8 of pregnancy. Through bioinformatic analysis of the data, our study contributes to an increase in the knowledge on the molecular mechanisms underlying regional decidualization in mice.

Materials and methods

Sample collection

Natural pregnancy model was established using CD-1 mice by co-caging adult females with fertile males. The day of the vaginal plug was designated as day 1 of pregnancy. The uterine segments of implantation sites were obtained on day 8 of pregnancy ($n=3$). Embryonic tissues were removed under a stereomicroscope. A complete removal was confirmed in sequential frozen sections. The embryo-free uterine segments were cut into halves to separate the mesometrial side (M) and the anti-mesometrial side (AM) of uterus. In addition to natural pregnancy, an artificial decidualization model was also established. In this model, female mice were mated with vasectomized males to induce pseudo-pregnancy. Intrauterine injection of 10 μ L sesame oil was performed to mimic the stimulation of the embryo on day 4 of pseudo-pregnancy. Uterine samples were collected on day 8 of pseudo-pregnancy ($n=3$). All collected samples were then snap-frozen in liquid nitrogen and stored at -80°C until use. All animal procedures were approved by the Institutional Animal Care and Use Committee of South China Agricultural University.

RNA-seq

Total RNA was extracted with the TRIzol reagent (Invitrogen). The RNA quality control parameters were A260/A280 ratio >1.8 , A260/A230 ratio >2.0 and RIN (RNA integrity number) value (Schroeder *et al.* 2006) >7.0 . The TruSeq RNA sample preparation kit (Illumina) was used to generate RNA-seq libraries following the manufacturer's protocol. High-throughput sequencing was conducted on the Illumina HiSeq 2500 system. Raw data were aligned to mouse genome (UCSC mm9) using TopHat with default options (Trapnell *et al.* 2009). The aligned reads were then provided as an input for the HTSeq count (Anders *et al.* 2015) to calculate read counts for each gene in the RefSeq database release 77 (O'Leary *et al.* 2016). Differential expression analysis was identified using the DESeq package (Anders & Huber 2010). Differentially expressed genes were chosen according to the criteria of fold-change >2 and adjusted P value <0.01 .

Validation by quantitative RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen). The cDNA was synthesized using the PrimeScript reverse transcriptase reagent kit (TaKaRa). Quantitative RT-PCR was carried out using the SYBR Premix Ex Taq kit (TaKaRa) on the Rotor-Gene 3000A system (Corbett Research). The Rpl7 served as the reference gene for normalization. Primer sequences were listed in Supplementary Table 1 (see section on supplementary data given at the end of this article).

Gene ontology (GO) analysis

GO analysis was performed by using the DAVID tool (Huang *et al.* 2007). GO terms with a gene count <10 were excluded from further analysis. The significance cutoff for

adjusted P value (the false discovery rate method) was set at 0.01. Redundant GO terms were removed manually.

Gene network analysis

The gene network was generated using the STRING database, v10.0 (Szklarczyk *et al.* 2015). The minimum combined score was set to 0.9. The Cytoscape software was applied for visualization and analysis of the gene network. The degree distribution was analyzed using the Cytoscape plugin Network Analyzer (Assenov *et al.* 2008). The degree threshold value for hub genes was the mean plus two standard deviations.

Analysis of transcription factor-binding sites (TFBS)

The putative promoter regions (1 kb upstream of transcription start site) were retrieved from the UCSC genome browser (Speir *et al.* 2016). The TESS software, version 6.0 (Schug 2008), was used to search for matches of position-weight matrices (PWM) available in the TRANSFAC database (Wingender *et al.* 1996). The cutoff value for relative score was set at 0.9. Using all genes in the genome as the background, a hypergeometric test with Benjamini–Hochberg multiple test correction was conducted using in-house PERL scripts. In the end, adjusted P value <0.01 was used as the significance threshold to identify enriched transcription factors.

Statistical analysis

Statistical analyses were performed in MATLAB 7.5 (MathWorks). Statistical significance of paired t -test was set at P value <0.05 .

Results

Transcriptomic differences at anti-mesometrial region compared to mesometrial region

To assess the molecular bases of regional differences in decidualization, we performed RNA-seq on anti-mesometrial (AM) region and mesometrial (M) region of mouse uterus on day 8 of pregnancy, with three biological replicates respectively (Fig. 1A). Differentially expressed genes were identified with the DESeq package using the following criteria: fold change >2 and adjusted P value <0.01 . As a result, 811 genes were upregulated and 612 genes were downregulated in the AM region compared to those in the M region respectively (Fig. 1B) (Supplementary Table 2). To validate the RNA-seq data, a total of 16 genes with various fold changes (non-changed: Alpl; upregulated: Ldha, Rrm2, Hand2, Dio3, Cryab, Prl3a2, Prl3c1 and Prss29; downregulated: Gzma, Wnt6, Wnt5a, Nr2f1, Fn1, Vim and Ldha) were selected and validated by quantitative RT-PCR (qRT-PCR). Although there was variation in fold changes between qRT-qPCR and RNA-seq, the expression patterns were coincident between these two techniques (Fig. 1C), indicating a high quality of our RNA-seq data.

Figure 1 Transcriptomic analysis of regional differences in decidualization of the mouse uterus. (A) A flowchart of the experimental design. M, mesometrial side; AM, anti-mesometrial side; TF, transcription factor. (B) Volcano plot for the comparison between AM and M samples. The cutoff values fold change >2 and adjusted P value <0.01 were utilized to identify differentially expressed genes. Non-changed genes were shown in blue color. Red color is indicative of upregulated genes and green is indicative of downregulated genes. (C) Validation of selected genes identified by RNA-Seq using qRT-PCR. Fold-change values determined by both RNA-seq and qRT-PCR were presented as the mean $\pm$ S.D. $n=3$.

Characterizing differentially expressed genes by gene ontology analysis

Gene ontology (GO) analysis of differentially expressed genes was performed to infer the functional consequences of their regional expression pattern. Over-represented GO terms, grouped in the three categories, biological process (BP), cellular component (CC) and molecular function (MF), were identified using the DAVID tool. The significance cutoff for FDR was set at 0.01. For upregulated genes, there were a total of 18 over-represented terms (Fig. 2A). In the BP category, 4 GO terms were significantly over-represented, namely ion transport, lipid catabolic process, cell adhesion and epithelial cell differentiation. The over-represented GO terms under the CC category were extracellular region, plasma membrane and cell–cell junction. With respect to the MF category, the over-represented GO terms were calcium ion binding, substrate-specific channel activity, alkali metal ion binding, channel activity, passive transmembrane transporter activity, serine hydrolase activity, serine-type endopeptidase activity, phosphoric diester hydrolase activity, serine-type peptidase activity, ion binding and phospholipase activity. In contrast, for downregulated genes, a total of 16 GO terms were over-represented (Fig. 2B), including lymphocyte activation, immune response, lymphocyte differentiation, cell adhesion, regulation of cytokine production, immune effector process, cell morphogenesis, hemopoiesis and cell morphogenesis involved in differentiation in the BP category, plasma membrane, proteinaceous extracellular matrix, extracellular region, cell surface and external side of plasma membrane under the CC category, and endopeptidase activity and calcium ion binding from the MF category. Only a small portion of over-represented GO terms (cell adhesion,

plasma membrane, extracellular region and calcium ion binding) were shared by both upregulated genes and downregulated genes, indicating that genes selectively enriched in these two compartments were functionally different.

Identification of hub genes by network analysis

To develop a thorough picture of differentially expressed genes at the systems level, gene network analysis was performed using integrative gene–gene interaction data from the STRING database. The reconstructed gene network for upregulated genes contained 56 genes and 184 gene–gene interactions (Fig. 3A), whereas the reconstructed gene network for downregulated genes consisted of 62 genes and 117 gene–gene interactions (Fig. 3B). Degree distribution analysis showed that both networks followed a power-law distribution and therefore belonged to scale-free small world networks (Barabasi & Oltvai 2004). Small world networks have the particular feature that some nodes, known as hub genes, are highly connected compared with others. Using a defined threshold value, we identified 4 hub genes (Plcb1, Kng1, Kng2 and Rhoc) for the network of upregulated genes and 2 hub genes (Vav1 and Gng2) for the network of downregulated genes. These genes represent functionally important genes due to their key positions in the networks and thus deserve further investigation.

Regulatory mechanisms revealed by the analysis of transcription factor-binding sites

Transcription factors are key regulators of gene expression. We extracted proximal promoter sequences

Figure 2 Gene ontology (GO) analysis of differentially expressed genes. (A) Significant GO terms associated with upregulated genes. (B) Significant GO terms associated with downregulated genes. The enrichment test was performed by using the DAVID tool and the significance cutoff for adjusted *P* value was set at 0.01. The number on the top of each bar is gene count. GO terms are arranged in 3 categories: biological process (BP), cellular component (CC) and molecular function (MF) respectively.

for all differentially expressed genes. Transcription factor-binding sites were predicted using the TESS software configured with position-weight matrices (PWM) from the TRANSFAC database. Upregulated genes and downregulated genes were separately tested for over-representation of transcription factor-binding sites. For upregulated genes, the binding sites of SREBP-1 (M00749), NF-kappaB (M00052), STAT6 (M00500), MAZ (M00649), CAC-BP (M00720) and LBP-1 (M00644) were significantly over-represented. For downregulated genes, only the SREBP-1 (M00749)-binding sites were significantly over-represented (Fig. 4). This analysis

provides clues to the regulatory mechanisms underlying regional decidualization of mouse uterus.

Global comparison between natural pregnancy and artificial decidualization

In mice, hormonally primed uterus can be stimulated by mechanical means (e.g. sesame oil) to trigger decidualization in the absence of an embryo (Ledford *et al.* 1976). The mechanically decidualized endometrium, known as the deciduoma, is morphologically similar to decidualization in natural pregnancy. To determine whether the observed regional decidualization in mouse uterus during natural pregnancy is dependent on the embryo, AM and M samples from artificially decidualized uterus were collected and subjected to RNA-seq. Whole-transcriptome Pearson correlation analysis demonstrated that natural pregnancy and artificial decidualization were highly similar for both AM and M samples (Fig. 5A). Differentially expressed genes were identified using the same criteria as described previously, leading to the discovery of 1206 upregulated genes and 1122 downregulated genes in AM region compared to those in M region (Supplementary Table 3). Among these genes, 578 upregulated genes and 517 downregulated genes were shared with natural pregnancy (Fig. 5B). To gain a systematic comparison, unsupervised hierarchical clustering analysis was conducted on the union of differentially expressed genes identified in natural pregnancy plus differentially expressed genes identified in artificial decidualization (Fig. 5C). The branching pattern illustrated in the dendrogram confirmed the transcriptomic similarity between natural pregnancy and artificial decidualization. In addition, results from qRT-PCR performed on a selected set of genes confirmed the consistency of gene expression pattern between natural pregnancy and artificial decidualization model (Supplementary Fig. 1). Taken together, these results suggest that regional decidualization also occurs in artificial decidualization in a similar way to natural pregnancy. Nevertheless, we did identify a small set of genes that were inconsistently expressed between artificial decidualization and natural pregnancy (Fig. 6A) (Supplementary Table 4). Two genes (Prap1 and Spint2) that were upregulated in natural pregnancy but downregulated in artificial decidualization model were validated using qRT-PCR (Fig. 6B). These genes were likely under the direct or indirect control of embryo-derived paracrine signals (Kashiwagi *et al.* 2007, McConaha *et al.* 2011).

Discussion

Although regional differences in mouse decidualization have been recognized for decades, the molecular mechanisms remain understudied. The present study

Figure 3 Gene network analysis of differentially expressed genes. (A) The network underlying upregulated genes. (B) The network underlying downregulated genes. This analysis was performed by using the STRING software. Nodes represent genes and edges represent gene-gene interactions. The diameter of each node is proportional to its degree value. This graph was generated using the Cytoscape software. The degree distribution of the network is shown as an inset. The degree distribution follows a power law distribution.

reports the first application of RNA-seq to characterize the transcriptomic differences between the anti-mesometrial (AM) region and the mesometrial (M) region of mouse uterus on day 8 of pregnancy. Implantation occurs on day 5 of pregnancy. The embryo does not

invade into the uterus until day 6. On day 8, the whole uterus, from AM region to M region, is in contact with the embryo. In response to embryo, the surrounding decidua is well developed. Placentation initiates on day 8. Therefore, we chose day 8 in this study as a crucial point for decidualization and placentation. We identified a total of 1423 differentially expressed genes. Quantitative RT-PCR (qRT-PCR) analysis demonstrated that the expression trend of selected genes was consistent with RNA-seq. This is in line with previous studies indicating that RNA-seq is a reliable and reproducible method to evaluate RNA expression (Mortazavi *et al.* 2008). Throughout the study, the CD-1 mouse strain was used. Compared to inbred mice, the outbred CD-1 mouse is easy to maintain and handle and reproduce quickly. Most importantly, the implantation rate is very stable between experiments. In our previous study, we demonstrated that female fertility in mice is highly variable among inbred strains (Liu *et al.* 2016). Therefore, strain-dependent differences in uterine gene expression may exist. However, the extent of differences has not been reported in literature yet.

Previously, using text mining, we compiled a list of 287 known genes associated with mouse decidualization and 286 genes associated with mouse decidualization, with an overlap of 111 genes shared by both species (Liu & Wang 2015). According to our RNA-seq data, 59 genes were differentially expressed: 33 genes were upregulated and 26 genes were downregulated in AM region compared to those in the M region (shown in Supplementary Table 5). These data suggest that regional differences in gene expression are relatively common

Figure 4 Analysis of transcription factor-binding sites. (A) Transcription factor analysis using the upregulated genes and downregulated genes respectively. (B) The sequence logo display for all transcription factors whose binding sites are significantly enriched.

Figure 5 Global comparison of the gene expression changes in artificial decidualization (AD) model against natural pregnancy (NP). (A) Mean Pearson correlation coefficients comparing the global gene expression changes between AD and NP. (B) Venn diagrams showing the number of differentially expressed genes using the criteria: fold change >2 and adjusted P value <0.01 . (C) Cluster dendrogram of the union gene set of differentially expressed genes in NP and differentially expressed genes in AD. The Pearson correlation distance measure and the average linkage clustering algorithm were used. The plot was generated by using bioinformatics toolbox 3.3 of MATLAB (MathWorks).

during mouse decidualization. Prolactin-related protein (Prl8a2) and alkaline phosphatase (Alpl) are two well-known biomarkers of decidualization in mice (Finn & Hinchliffe 1964, Orwig *et al.* 1997, Soares *et al.* 1998, Li *et al.* 2007). Interestingly, although Alpl was evenly expressed, Prl8a2 was approximately 16-folds higher in AM region compared to that in M region. This result was confirmed by qRT-PCR. Thus, Alpl is a mutual biomarker for decidualization in AM region and M region, whereas Prl8a2 is a selective biomarker for decidualization in AM region.

Because of the anatomy differences and eccentric embryo implantation in the mouse (Lee & DeMayo 2004), our data cannot be extrapolated to the human in a straightforward way. Unlike the case of mouse, decidualization is initiated spontaneously in the secretory phase of menstrual cycle in human. If embryo undergoes implantation, decidualization continues;

otherwise, menstruation occurs. Previously, a study has demonstrated that perivascular endometrial stromal cells are programmed to mount a distinctive decidual response (Murakami *et al.* 2014). Given that the M region of mouse uterus is enriched in blood vessels, we suspected that the majority of human perivascular endometrial stromal cells may be similar to the AM region of mouse uterus, whereas the perivascular endometrial stromal cells are equivalent to the M region of mouse uterus. Further studies are needed to verify this hypothesis.

Gene ontology (GO) analysis provided us important clues to the functional consequences of differentially expressed genes. As expected, upregulated genes were generally involved in cell metabolism and differentiation, whereas downregulated genes were associated with lymphocyte themes and immune response. When trophoblast invasion triggers by inflammatory response in the AM side, immune cells may be recruited into uterus from the vessel-enriched M side. Uterine dendritic cells (DC) are crucial for decidual formation and placental development (Krey *et al.* 2008, Plaks *et al.* 2008). Uterine regulatory T cells (Treg) contribute to the maintenance of tolerance to paternal antigens in fetal cells (Samstein *et al.* 2012). Uterine natural killer cells (uNK) and macrophages participate in the reconstruction of uterine vasculature, which is fundamental to placentation and gestation maintenance (Nagamatsu & Schust 2010, Ratsep *et al.* 2015). Our results supported the theory that decidualization in the AM region is involved in the accommodation of embryo implantation, and decidualization in the M region contributes to the preparation of placentation (Dey *et al.* 2004).

Network analysis was performed to identify hub genes for upregulated genes and downregulated genes. The 4 hub genes for network of upregulated genes were Plcb1 (phospholipase C beta 1), Kng1 (kininogen 1), Kng2 (kininogen 2) and Rhoc (ras homolog gene family member C). The 2 hub genes for network of downregulated genes were Vav1 (vav 1 oncogene) and Gng2 (guanine nucleotide-binding protein gamma 2). Plcb1, Rhoc, Vav1 and Gng2 are related to G proteins. Kng1 and Kng2 have multiple effects in the vascular system (Sharma 2006). These hub genes are likely more important than other genes due to their key positions in the network.

Transcription factors are key regulators of gene expression. Interestingly, we found that SREBP-1 (sterol regulatory element-binding protein 1) is a commonly over-represented transcription factor for both upregulated and downregulated genes. SREBP-1 belongs to a family of basic helix-loop-helix-leucine zipper (bHLHLZ) transcription factors (Yokoyama *et al.* 1993). Known target genes of SREBP-1 are involved in cholesterol biosynthesis and transport (Brown & Goldstein 1997, 1999). Additionally, we found that

Figure 6 Identification of inconsistently expressed genes between artificial decidualization (AD) and natural pregnancy (NP). (A) Heatmap of inconsistently expressed genes. Candidate genes were selected based on the following criteria: ratio >2 and adjusted *P*-value <0.01. (B) Validation of two representative genes by qRT-PCR. Data were presented as the mean $\pm$ S.D. *n*=3. **P*<0.05.

the binding sites of NF-kappaB, STAT6, MAZ, CAC-BP and LBP-1 were significantly over-represented in the upregulated genes. NF-kappaB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls immune response, cytokine production and cell survival (Baldwin 1996). NF-kappaB is activated at the implantation site of the mouse uterus during early pregnancy (Nakamura *et al.* 2004). In human endometrial stromal cells, activated NF-kappaB protects cells from apoptosis during decidualization (Fluhr *et al.* 2013). STAT6 (signal transducer and activator of transcription 6) and its family member STAT3 share the same binding motif (Mascareno *et al.* 1998). STAT3 is a key regulator of mouse decidualization. Conditional ablation of STAT3 in uterus results in a failure of decidualization reaction (Lee *et al.* 2013). CAC-BP (CAC-binding protein) binds CAC box and regulates gene expression in a cis manner. MAZ (Myc-associated zinc finger protein) is a transcription factor with dual roles in transcription initiation and termination. One

target of MAZ is MYC (v-myc avian myelocytomatosis viral oncogene homolog) (Bossone *et al.* 1992). LBP-1 (upstream binding protein 1) is a member of the NTF (neurogenic element-binding transcription factor) family of transcription factors. Currently, little is known about the role of SREBP-1, CAC-BP, MAZ and LBP-1 in regulating uterine gene expression. Hence, these four transcription factors deserve further investigation.

Is the observed regional decidualization in mouse uterus during natural pregnancy dependent on the embryo? To answer this question, we used the artificial decidualization model, in which the sesame oil was used as a mimic of the embryo (Ledford *et al.* 1976). Whole-transcriptome Pearson correlation analysis revealed that regional decidualization also occurs in artificial decidualization. In artificial decidualization model, we identified 1206 upregulated genes and 1122 downregulated genes. Among these genes, 578 upregulated genes and 517 downregulated genes were shared with natural pregnancy. Results from

qRT-PCR performed on a selected set of genes confirmed the consistency of gene expression pattern between natural pregnancy and artificial decidualization model. Nevertheless, we did identify a small set of genes that were inconsistently expressed between artificial decidualization and natural pregnancy. For example, Prap1 and Spint2 were upregulated in natural pregnancy but downregulated in artificial decidualization model. The regulatory mechanism and functional consequence for these genes are unknown.

In conclusion, in the present study, using RNA-seq, we compared the transcriptomic differences between the AM region and the M region of mouse uterus on day 8 of pregnancy. Our study provides a valuable resource for in-depth understanding of the molecular mechanisms underlying regional decidualization in mice.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0486>.

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.

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