Microarray assessment of the influence of the conceptus on gene expression in the mouse uterus during decidualization

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Abstract

During pregnancy in several species including humans and rodents, the endometrium undergoes decidualization. This process of differentiation from endometrial to decidual tissue occurs only after the onset of implantation in mice. It can also be artificially induced causing the formation of decidualoma tissue. The purpose of this study was to compare the gene expression profile of the developing decidua in pregnant mice with the decidualoma formed after artificial induction in an effort to identify conceptus-influenced changes in uterine gene expression during decidualization. We induced decidualization artificially by transferring blastocyst-sized ConA-coated agarose beads into the uterus on day 2.5 of pseudopregnancy. Recently published work has found this model to be more 'physiological' than other methods. Total RNA was isolated from blastocyst and bead-induced 'implantation' sites of the uteri of day 7.5 pregnant (decidua) and pseudopregnant (decidualoma) mice respectively. This RNA was then used for microarray analysis using Mouse Illumina BeadArray chips. This analysis revealed potential differential mRNA levels of only 45 genes between the decidua and bead-induced decidualoma tissues. We confirmed the differential mRNA levels of 31 of these genes using quantitative RT-PCR. Finally, the level and localization of some of the mRNAs for select genes (*Aldh3a1*, *Bcmo1*, *Guc2b*, and *Inhbb*) identified by our microarray analysis were examined in more detail. This study provides the identity of a small set of genes whose expression in the uterus during decidualization may be influenced by molecular signals from the conceptus.

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Introduction

In mammals, implantation begins with the interaction of the conceptus with the uterine epithelium and ends in the formation of the definitive placenta. In some species such as humans and rodents, the endometrium undergoes decidualization during implantation (reviewed in Krehbiel (1937), Tachi & Tachi (1974), Abrahamsohn & Zorn (1993), Abrahamsohn *et al.* (2002) and Gellersen *et al.* (2007)). Decidualization is the process where the endometrial tissue changes into decidual tissue and is critical in the establishment of pregnancy. The changes that occur during decidualization include differentiation of the endometrial fibroblast-like cells into epithelioid-like decidual cells, extracellular matrix remodeling, apoptosis, angiogenesis, and changes in immune cell populations. The endometrial tissue is believed to provide a key environment required for the developing conceptus until the functional placenta is formed. Although only select species are said to exhibit uterine decidualization, similar changes have been suggested for other species including those where implantation is

superficial (Mossman 1937, Kellas 1966, King *et al.* 1980, MacIntyre *et al.* 2002, Johnson *et al.* 2003, Joyce *et al.* 2005). However, more work is required to characterize the similarities and differences between mammalian species with quite different types of implantation.

The decidualization response can be initiated by different means depending on species. For example, in humans, decidualization begins near the end of the secretory phase of the menstrual cycle (Gellersen *et al.* 2007). This is driven by the effects of progesterone from the ovary, and if pregnancy is not obtained, menstruation occurs. However, if pregnancy is obtained, decidualization continues as the human conceptus undergoes implantation. In the rodent uterus, the situation is slightly different. Although there is no doubt that it is also progesterone dependent in rodent species (Psychoyos 1973), decidualization will not begin unless there is an 'implantation stimulus'. This stimulus is likely physical in nature (Kennedy *et al.* 2007). The requirement for a physical implantation stimulus in mice and rats is exemplified by many models, where decidualization is

induced artificially in the uteri of pseudopregnant, pregnant, or ovariectomized hormone-sensitized animals. Methods to artificially induce decidualization have ranged from quite traumatic stimuli such as electrical shock (Loeb, 1908) to those that are believed to be more physiological such as intraluminal instillation of sesame oil (Deb *et al.* 2006). In all cases, the tissue that forms in response to an artificial stimulus is called a deciduoma to discern it from the decidua that forms in pregnant animals.

Previous work has suggested that the mammalian conceptus may secrete paracrine factors that can regulate endometrial gene expression during decidualization (reviewed in Herington & Bany (2009)). Clear examples of this exist in several species. For example, it is certain that human chorionic gonadotrophin and interferon τ are secreted by the human and bovine conceptuses respectively, and they regulate gene expression in the endometrium during implantation (Thatcher *et al.* 2001, Licht *et al.* 2007, Spencer *et al.* 2007, Sherwin *et al.* 2008). Recently, we and others have sought to find such conceptus factors as well as targeted endometrial genes in the mouse (Austin *et al.* 2003, Bany & Cross 2006, Kashiwagi *et al.* 2007, Herington *et al.* 2009). The approach used was to compare uterine gene expression between the decidua and oil-induced deciduomas. This approach seemed reasonable at the time as the oil-induced deciduoma models were the most commonly used. As a consequence of this work, a large number of genes were identified whose expression in the endometrium may be under the direct or indirect control of conceptus-derived paracrine signals. Recently, however, several different artificial models of decidualization were compared and suggest that a Concanavalin A-coated agarose bead model in pseudopregnant mice may have several advantages over the oil-induced deciduoma models (Herington *et al.* 2009). For example, a focal deciduogenic stimulus is provided by the bead, and the localized decidualization that occurs is similar to that of pregnancy. The deciduogenic stimulus provided by oil infusion into the lumen is along the entire length of the uterine horn. Therefore, the bead-induced decidualization model was used in this study, as it may be a more physiologically relevant. In this study, we performed a large-scale analysis to determine what genes may be differentially expressed in the uteri of pseudopregnant mice undergoing bead-induced decidualization compared with that of the pregnant uterus during decidualization using the Illumina platform.

Results

Microarray analysis

To determine the potential effect of the conceptus on uterine gene expression during decidualization, we compared the global mRNA levels between RNA

samples from day 7.5 implantation site (IS) decidua and bead-induced deciduoma (BID) uterine segments using Illumina BeadChip microarray analysis. Of the 46 633 probes, signals for 13 063 were detected significantly above background in all samples. Analysis of the data suggested that 53 of these showed a significant difference in the level of hybridization signals between the two tissue types. Of these 53 probes, five (probe IDs 103390487, 100050500, 101340040, 2850731, and 2100170) could not be annotated or mapped to the mouse genome and thus were removed from further analysis. The remaining 48 represent the mRNAs for 45 different genes, of which 27 (Table 1) and 18 (Table 2) had greater hybridization signals in the BID and IS segment RNA samples respectively. A visualization of the relative level of the hybridization signals of these probes is provided in a heat map found in Fig. 1. Full annotation of the 45 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 revealed significant overrepresentation of GO terms for biological processes and molecular function (see Supplementary Table 2, see section on supplementary data given at the end of this article).

As previously shown (Bany & Cross 2006, Herington *et al.* 2009), hybridization signals of several markers of decidualization including *Bmp2*, *Bmp8a*, *Fkbp5*, and *Hand2* were not significantly different between the IS and BID segments. Furthermore, since they are the cells of the conceptus in most intimate contact with the endometrium, it is also notable that hybridization signals of several trophoblast cell-specific mRNAs (Simmons *et al.* 2008) including *Tpbpa*, *Tpbpb*, several prolactin-related genes (*Prl2a1*, *Prl3b1*, *Prl5a1*, *Prl7a1*, *Prl7a2*, *Prl7c1*, *Prl7d1*, and *Prl8a1*), and *Hand1* were not detected above background. This suggests that the samples used for the microarray analysis had negligible levels of contaminating trophoblast and ectoplacental cone tissues. Notably, although originally believed to be trophoblast specific and expressed only later in pregnancy (Hwang *et al.* 2000), *Prl8a9* mRNA was detected in all microarray samples in this study. This confirms the recent work (Simmons *et al.* 2008) showing that it is expressed at low levels in the maternal decidual cells on day 7.5.

Independent verification of microarray results

Of the 27 genes examined further, we verified differential expression of 23 between the day 7.5 IS and BID segment tissues using quantitative real-time PCR (qRT-PCR; Table 3). The steady-state mRNA levels of 8 of these 23 genes were significantly greater in the IS segment tissue, while that of the remaining 15 were significantly greater in the BID segment tissue. The expression of four additional genes identified as differentially expressed in the microarray analysis was also studied in more detail as described below.

Table 1 Genes whose steady-state mRNA levels are significantly lower in the day 7.5 decidua than in the day 7.5 bead-induced deciduoma (BID). Probe ID, Illumina Probe identification number; Fold, average decidual compared to BID tissue levels.

Symbol	Description	Probe ID	Fold
<i>Ear2</i>	Eosinophil-associated, RNase A family, member 2	2360471	0.08
<i>Ltf</i>	Lactotransferrin	6770364	0.11
<i>Ear2</i>	Eosinophil-associated, RNase A family, member 2	5670239	0.12
<i>Padi1</i>	Peptidylarginine deiminase, type I	5080270	0.12
<i>Klk1b27</i>	Kallikrein 1-related peptidase b27	5900632	0.17
<i>Dctn1</i>	Dynactin 1	1450056	0.19
<i>Klk1b5</i>	Kallikrein 1-related peptidase b5	2350528	0.19
<i>1190003J15Rik</i>	RIKEN cDNA 1190003J15 gene	540408	0.19
<i>Klk1</i>	Kallikrein 1	104730373	0.20
<i>Klk1b26</i>	Kallikrein 1-related peptidase b26	101850092	0.21
<i>Inhbb</i>	Inhibin beta-B	2680092	0.21
<i>Klk1b3</i>	Kallikrein 1-related peptidase b3	630348	0.21
<i>Adrbk2</i>	Adrenergic receptor kinase, beta 2	102970520	0.22
<i>Guca2b</i>	Guanylate cyclase activator 2b (retina)	4200014	0.22
<i>Klk1b27</i>	Kallikrein 1-related peptidase b27	2810008	0.22
<i>6720418B01Rik</i>	RIKEN cDNA 6720418B01 gene	103870341	0.24
<i>Klk1</i>	Kallikrein 1	4010706	0.24
<i>Fcgbp</i>	Fc fragment of IgG-binding protein	102570725	0.25
<i>2310043J07Rik</i>	RIKEN cDNA 2310043J07 gene	102230139	0.25
<i>Lcn2</i>	Lipocalin 2	2510112	0.25
<i>Tmprss4</i>	Transmembrane protease, serine 4	1050402	0.25
<i>Erv3</i>	Endogenous retroviral sequence 3	3130338	0.25
<i>2310033E01Rik</i>	RIKEN cDNA 2310033E01 gene	102630524	0.26
<i>9030607L20Rik</i>	RIKEN cDNA 9030607L20 gene	106020102	0.27
<i>2900076A13Rik</i>	RIKEN cDNA 2900076A13 gene	102900722	0.27
<i>Taok2</i>	TAO kinase 2	2810484	0.29
<i>F830028O17Rik</i>	RIKEN cDNA F830028O17 gene	102480324	0.29
<i>Col3a1</i>	Collagen, type III, alpha 1	6420273	0.30
<i>Foxp4</i>	Forkhead box P4	107050112	0.32
<i>Tmem14c</i>	Transmembrane protein 14C	3940193	0.34

Expression of select genes involved in retinoic acid homeostasis

The beta-carotene 15,15'-monooxygenase (*Bcmo1*) gene encodes a protein (BCMO1), which catalyzes the conversion of β -carotene to retinal and is important for the production of retinoic acid (Barua & Olson 2000, Hessel *et al.* 2007; Fig. 2A). Since this gene appeared to be differentially expressed on day 7.5 in our microarray analysis, we further examined uterine expression during decidualization of this and related genes involved in retinoic acid metabolism. Initially, we verified significant ($P < 0.01$) differential expression by qRT-PCR (Fig. 2B). Next, we examined *Bcmo1* expression in the pregnant mouse uterus on days 3.5–8.5 (Fig. 2C). *Bcmo1* mRNA levels were low in the uterus on days 3.5–4.5 of pregnancy. However, the levels significantly ($P < 0.01$) increased on days 5.5–8.5 in IS segment compared with non-implantation site (NIS) segment. To further define the expression differences during decidualization, we determined the levels of *Bcmo1* mRNA in IS segment compared to BID segment of the uterus on days 4.5–8.5 of pregnancy and pseudopregnancy respectively (Fig. 2D). The mRNA levels were significantly ($P < 0.01$) greater in the IS segment than in the BID segment only on day 7.5.

To determine the localization of expression, we attempted to localize *Bcmo1* mRNA in the uterus on days 5.5–8.5 of pregnancy or pseudopregnancy by *in situ* hybridization. No hybridization signals were detected with sense probes in any of the tissues as well as with antisense probes in uterine tissues from day 5.5 and in NIS or non-bead (NB) segments on days 6.5–8.5 (data not shown). On day 6.5, for both IS (Fig. 3A and B) and BID (Fig. 3C and D) segments, *Bcmo1* mRNA was localized to a small area lateral to the implanting conceptus and bead respectively. On day 7.5, the signals spread mesometrially to include maternal tissue surrounding the blood islands that form in the lateral mesometrial area in the IS (Fig. 3E and F). On the other hand, only the lateral staining was observed in the day 7.5 BID segments (Fig. 3G and H). Finally, on day 8.5, *Bcmo1* mRNA signals were detected in the maternal tissue completely surrounding the conceptus (Fig. 3I and J). However, hybridization signals were mainly detected in the maternal tissue lateral to the bead stimulus in the BID segments on day 8.5 of pseudopregnancy (Fig. 3K and L).

The microarray data indicated that several genes involved in retinoic acid homeostasis and action were expressed at similar levels in the day 7.5 IS and BID tissues. To verify this and determine the occurrence of false-negative microarray findings, we independently

Table 2 Genes whose steady-state mRNA levels are significantly greater in the day 7.5 decidua than in the day 7.5 bead-induced decidualoma (BID). Probe ID, Illumina Probe identification number; Fold, average decidual compared to BID tissue levels.

Symbol	Description	Probe ID	Fold
<i>Capn6</i>	Calpain 6	1740168	2.54
<i>Hist1h2ah</i>	Histone cluster 1, H2ah	3710333	2.89
<i>Cald1</i>	Caldesmon 1	103870092	2.96
<i>Crybg3</i>	Beta-gamma crystallin domain containing 3	1770048	3.00
<i>Cpd</i>	Carboxypeptidase D	5860685	3.01
<i>St8sia4</i>	ST8 alpha-N-acetylneuraminide alpha-2, 8-sialyltransferase 4	3060215	3.06
<i>Pik3r3</i>	Phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3 (p55)	100130097	3.10
<i>Ankrd17</i>	Ankyrin repeat domain 17	2120402	3.14
<i>Pfdn4</i>	Prefoldin 4	4010286	3.37
<i>Zfp800</i>	Zinc finger protein 800	2350253	3.37
<i>Mtap7d2</i>	MAP7 domain containing 2	130471	3.51
<i>Stmn2</i>	Stathmin-like 2	6220181	3.57
<i>Aldh3a1</i>	Aldehyde dehydrogenase family 3, subfamily A1	580095	3.58
<i>Usmg5</i>	Upregulated during skeletal muscle growth 5	3710181	4.27
<i>Bcmo1</i>	Beta-carotene 15,15'-monooxygenase	4780121	4.94
<i>Cst9</i>	Cystatin 9	2230706	6.73
<i>Prps2</i>	Phosphoribosyl pyrophosphate synthetase 2	3140707	7.20
<i>2900060B14Rik</i>	RIKEN cDNA 2900060B14 gene	102810039	9.59

measured expression levels using qRT-PCR for a small number of these targets. The aldehyde dehydrogenase 1a3 gene (*Aldh1a3*) encodes a protein that plays a critical role of converting retinal to retinoic acid (Fig. 2A). As shown in Fig. 2E, we confirmed that *Aldh1a3* mRNA levels were not significantly ($P>0.05$) different between the IS and BID segments on day 7.5. The *Rdh10* gene encodes a protein that can oxidize retinol into retinal and thus is important in retinoic acid homeostasis (Fig. 2A). As shown in Fig. 2F, we confirmed that retinol dehydrogenase 10 (*Rdh10*) mRNA levels were not significantly ($P>0.05$) different between the IS and BID segments on day 7.5. The retinol dehydrogenase 11 (*Rdh11*, previously called *Psdr1*) and 14 (*Rdh14*, previously called *Pan2*) genes encode proteins that can reduce retinal into retinol (Fig. 2A). We concluded that *Rdh11* (Fig. 2G) plus *Rdh14* (Fig. 2H) mRNA levels were similar between the IS and BID segments on day 7.5. Finally, the cytochrome P450 family 26 subfamily b polypeptide 1 (*Cyp26b1*) gene encodes a protein that degrades retinoic acid in tissues (White *et al.* 2000). We confirmed that mRNA levels did not differ between the IS and BID segments on day 7.5 (Fig. 2I). Interestingly, *Cyp26b1* mRNA levels were significantly greater in the IS segment than in the NIS segment of pregnant uteri on days 7.5 and 8.5 (Fig. 2J).

Expression of type 3 aldehyde dehydrogenases

The type 3a1 aldehyde dehydrogenase gene (*Aldh3a1*) encodes a protein (ALDH3A1) that is believed to be involved in the breakdown of products of medium-chained lipid peroxidation in the eye (Pappa *et al.* 2003). Since this gene appeared to be differentially expressed on day 7.5 in our microarray analysis, we further examined its expression in the uterus during decidualization. We confirmed that *Aldh3a1* mRNA levels were significantly greater ($P<0.05$) in the IS segment than in the BID segment on day 7.5 by qRT-PCR (Fig. 4A). In addition, we examined the expression in the pregnant mouse uterus on days 3.5–8.5 (Fig. 4B). The mRNA levels dramatically increased and were significantly greater ($P<0.05$) in the IS segment than in the NIS segment only on days 7.5 and 8.5. *Aldh3a1* mRNA

Figure 1 Heat map of differentially expressed genes between the day 7.5 decidua and decidualoma (BID). Red to black to blue indicates a gradient of high to low expression.

was localized in uterine sections from day 5.5 to 8.5 pregnant or pseudopregnant mice by *in situ* hybridization (Fig. 5). Hybridization signals were never detected in uterine tissues on day 5.5 and in NIS or NB segments on days 6.5–8.5 (data not shown). On day 6.5, for both IS (Fig. 5A and B) and BID (Fig. 5C and D) segments, *Aldh3a1* mRNA was localized to a small area just lateral to the implanting conceptus or bead. On day 7.5, the signals spread mesometrially to include maternal tissue surrounding the blood islands that form in the lateral mesometrial area in the IS (Fig. 5E and F) and BID (Fig. 5G and H) segments. Finally, on day 8.5, *Aldh3a1* mRNA signals were detected in the maternal tissue completely surrounding the conceptus (Fig. 5I and J). However, in the BID segments of the pseudopregnant mice, hybridization signals were only detected laterally in the mesometrial–anti-mesometrial border of the deciduoma tissue (Fig. 5K and L).

The type 3a2 aldehyde dehydrogenase gene (*Aldh3a2*) is closely related to *Aldh3a1* and is involved in oxidation of long- to medium-chained aliphatic aldehydes in the liver and thus may be involved in the detoxification of aldehydes produced by lipid peroxidation (Mitchell & Petersen 1989). The levels of *Aldh3a2* mRNA did not differ between the IS and BID segment tissues on day 7.5 of pregnancy and pseudopregnancy respectively (Fig. 4C). Furthermore, *Aldh3a2* mRNA levels did not differ between the NIS and IS segments except for day 7.5, where levels were significantly ($P<0.05$) higher in the IS segment than in the NIS segment (Fig. 4C).

Inhibin β gene expression

Members of the inhibin gene family encode proteins that make up subunits of inhibins and activins that play several roles in reproductive endocrinology (Ethier & Findlay 2001). We confirmed that inhibin β (*Inhbb*) mRNA levels were significantly greater in the BID segment than in the IS segment by qRT-PCR (Fig. 6A). To further characterize *Inhbb* expression in the uterus during decidualization, we next examined mRNA levels in the pregnant uterus during the peri-implantation period. *Inhbb* mRNA was detected at low levels in the uterus on day 3.5 of pregnancy prior to the onset of implantation (Fig. 6B). The levels did not significantly ($P>0.05$) change in NIS segments on days 4.5–8.5. However, on each of these days, there were significant ($P<0.05$) increases in *Inhbb* mRNA levels in the IS segment compared with those in the NIS segment of the pregnant uterus. Finally, to determine the localization of *Inhbb* expression in the IS and BID tissues, we used *in situ* hybridization (Fig. 6C–F). Hybridization signals were detected only in the vascular endothelial cells lining the blood islands in the mesometrial tissue adjacent to the implanting conceptus or bead. Notably, two different hybridization probe synthesis templates were generated using the primers shown in Supplementary

Table 3B, see section on supplementary data given at the end of this article. These two probes cover different regions of the *Inhbb* mRNA, and localization of hybridization signals using the two different antisense probes was identical (data not shown).

Follistatin (encoded by the *Fst* gene) binds to activins preventing their ability to activate target receptors (Ethier & Findlay 2001). Therefore, we examined the expression of the *Fst* gene more closely. Unlike *Inhbb*, microarray analysis indicates no difference in *Fst* and *Inhba* mRNA levels between the IS and BID segments on day 7.5. We confirmed that *Fst* mRNA levels did not differ significantly ($P>0.05$) between the BID and IS segments on day 7.5 of pregnancy by qRT-PCR (Fig. 6G). Further analysis of *Fst* mRNA levels in pregnant uteri showed that levels are significantly greater ($P<0.01$) in the IS segment than in the NIS segment on days 4.5–8.5 of pregnancy (Fig. 6H).

Characterization of guanylate cyclase activator 2 gene (*Guca2a* and *Guca2b*) expression

Guca2a and *Guca2b* genes encode secreted endocrine/paracrine hormones that bind to the same guanylate cyclase receptor and have well-characterized roles in controlling salt handling and fluid balance in the

Table 3 qRT-PCR verification of differential expression between the day 7.5 implantation (IS) and day 7.5 pseudopregnant bead-induced deciduoma (BID) segments.

Symbol	IS	BID	P value
11190003J15Rik	1.0 $\pm$ 0.12	7.0 $\pm$ 1.44	0.002
2310033E01Rik	1.0 $\pm$ 0.20	10.2 $\pm$ 2.69	0.001
2310043J07Rik	1.0 $\pm$ 0.20	3.5 $\pm$ 1.21	0.034
2900060B14Rik	1.0 $\pm$ 0.11	0.5 $\pm$ 0.05	0.003
9030607L20Rik	1.0 $\pm$ 0.17	7.9 $\pm$ 2.74	0.013
Adrbk2	1.0 $\pm$ 0.28	11.6 $\pm$ 3.61	0.003
Ankrd17	1.0 $\pm$ 0.20	1.4 $\pm$ 0.36	0.293*
Cald1	1.0 $\pm$ 0.18	0.3 $\pm$ 0.07	0.008
Col3a1	1.0 $\pm$ 0.17	2.3 $\pm$ 0.32	0.003
Cpd	1.0 $\pm$ 0.27	0.07 $\pm$ 0.02	0.014
Crybg3	1.0 $\pm$ 0.15	0.35 $\pm$ 0.07	0.004
Dctn1	1.0 $\pm$ 0.25	1.7 $\pm$ 0.24	0.081*
Ear2	1.0 $\pm$ 0.33	166.5 $\pm$ 20.00	0.000
Erv3	1.0 $\pm$ 0.27	1.5 $\pm$ 0.19	0.122*
Foxp4	1.0 $\pm$ 0.24	1.5 $\pm$ 0.18	0.114*
Ilk1	1.0 $\pm$ 0.18	10.2 $\pm$ 3.13	0.004
Ilk1b27	1.0 $\pm$ 0.21	42.5 $\pm$ 8.8	0.000
Lcn2	1.0 $\pm$ 0.09	7.4 $\pm$ 1.92	0.01
Ltf	1.0 $\pm$ 0.08	12.8 $\pm$ 3.45	0.002
Padi1	1.0 $\pm$ 0.05	12.8 $\pm$ 2.1	0.003
Pfdn4	1.0 $\pm$ 0.13	0.35 $\pm$ 0.13	0.006
Prps2	1.0 $\pm$ 0.11	0.3 $\pm$ 0.08	0.009
Stmn2	1.0 $\pm$ 0.10	0.5 $\pm$ 0.18	0.041
Taok2	1.0 $\pm$ 0.18	14.7 $\pm$ 4.84	0.002
Tmem14c	1.0 $\pm$ 0.15	2.2 $\pm$ 0.60	0.043
Tmprss4	1.0 $\pm$ 0.15	6.7 $\pm$ 1.11	0.000
Zfp800	1.0 $\pm$ 0.17	0.24 $\pm$ 0.06	0.002

Values represent the mean ($\pm$ S.E.M.; $N=4-6$) relative mRNA levels normalized to 18S rRNA levels. *Not significantly different.

Figure 2 qRT-PCR analysis of steady-state mRNA levels of select genes involved in retinoic acid metabolism. (A) Simplified schematic of the retinoic acid homeostasis pathways and the genes whose steady-state mRNA levels we examined. (B) Relative *Bcmo1* mRNA levels in day 7.5 bead-induced decidualoma (BID) segment compared with implantation site (IS) segment tissues. (C) Relative *Bcmo1* mRNA levels in the non-implantation site (NIS) segment compared with the IS segment on days 3.5–8.5 of pregnancy. (D) Relative *Bcmo1* mRNA levels in the BID segment compared with the IS segment on days 3.5–8.5 of pseudopregnancy and pregnancy respectively. Relative levels of (E) *Aldh1a3*, (F) *Rdh10*, (G) *Rdh11*, and (H) *Rdh14* between the BID and IS segments on day 7.5. (I) Relative *Cyp26b1* mRNA levels in the BID segment compared with the IS segment on day 7.5 of pseudopregnancy and pregnancy respectively. (J) Relative *Cyp26b1* mRNA levels in the NIS and IS segments on days 3.5–8.5 of pregnancy respectively. * $P < 0.05$ and ** $P < 0.01$. Bars represent mean $\pm$ S.E.M. ($N = 4$).

intestines and kidney (Sindic & Schlatter 2006a, 2006b, 2007). Microarray analysis indicated that *Guca2b*, but not *Guca2a*, mRNA levels differed between the BID and IS segments on day 7.5 of pseudopregnancy and pregnancy respectively. This was verified by qRT-PCR as shown in Fig. 7A and B where it was observed that the steady-state *Guca2b* mRNA levels were significantly greater (by ~ 4.5 -fold, $P < 0.001$) in the BID segment than in the IS segment tissues. Closer examination of pregnant uteri during the peri-implantation period revealed that *Guca2b* mRNA levels were low on day 3.5 of pregnancy (prior to the onset of implantation) compared with the days following the onset of implantation (Fig. 7C). After the onset of implantation,

Guca2b mRNA levels between the NIS segments were significantly ($P < 0.05$) greater than that of the IS segments only on day 8.5 and no other day examined. Finally, *Guca2b* mRNA levels in BID segment compared to IS segment on days 3.5–8.5 of pseudopregnancy and pregnancy are shown in Fig. 7D. On day 3.5, *Guca2b* mRNA levels were significantly ($P < 0.001$) greater in the pseudopregnant mice than in pregnant mice by ~ 11.5 -fold. On days 4.5–6.5, mRNA levels were not significantly different between the BID and IS segment tissues. Again, however, mRNA levels were significantly ($P < 0.05$) greater in the BID segment than in IS segment tissues on day 7.5 by fivefold. On day 8.5, this difference became approximately twofold ($P < 0.01$).

Figure 3 Localization of *Bcmo1* mRNA in implantation site (IS) and bead-induced deciduoma (BID) segments of pregnant and pseudopregnant mice. Photomicrographs of day 6.5 IS (A and B) or BID (C and D), day 7.5 IS (E and F) or BID (G and H), and day 8.5 IS (I and J) or BID (K and L) segments are shown. AM, anti-mesometrial side; C, conceptus; IS, implantation site; MM, mesometrial side. Numbers above scale bars are in microns. Representative of at least three independent samples. Global linear adjustments of the brightness and color level were made on the photomicrographs to more accurately represent what was observed on the slides under the microscope.

Assessing the potential role of giant cells

To determine the potential role of trophoblast giant cells in the differential expression of four of the genes found differentially expressed between the BID and IS segments by microarray and qRT-PCR analyses, we used mice carrying a null mutation in the *Hand1* gene. *Hand1*-deficient conceptuses do not have trophoblast giant cells, and they die around days 7.5–8.5 (Riley *et al.* 1998). As shown in Table 4, *Aldh3a1*, *Bcmo1*, *Guca2b*, and *Inhbb* mRNA levels did not differ in the IS segment tissue containing *Hand1* wild-type compared with those containing knockout conceptuses.

Assessing select genes previously found to be differentially expressed between the decidua and oil-induced deciduoma

Previous work indicated a great number of genes differentially expressed between the decidua and oil-induced deciduoma on day 7.5 of pregnancy and pseudopregnancy respectively (Kashiwagi *et al.* 2007). If the microarray data of this study indeed have a high false-negative rate, then many of the genes identified in the previously published microarray work may also be differentially expressed between the decidua and BID. Therefore, we assessed the differential expression of four additional random genes between the IS and BID tissues previously identified to be differentially expressed by Kashiwagi *et al.* (2007) between the decidua and oil-induced deciduoma. As shown in Table 5, *Dact1*, *Hoxb6*, *Nkg7*, and *Prss28* (Isp1-pending in Kashiwagi

et al. (2007)) mRNA levels did not differ between the BID and IS tissues on day 7.5 of pseudopregnancy and pregnancy respectively. Since *Prss28* was previously shown to be one of the genes more differentially expressed between the decidua and oil-induced deciduoma, we localized its expression using *in situ* hybridization. *Prss28* hybridization signals were detected in the glandular epithelial cells throughout day 7.5 pregnant uterus in the NIS (Fig. 8A) and IS (Fig. 8B and C) tissues. Similarly, *Prss28* hybridization signals were detected in the glandular epithelial cells throughout day 7.5 pseudopregnant uterus in the interbead (Fig. 8D) and BID (Fig. 8E and F) tissues. In both the pregnant and pseudopregnant uteri, more hybridization signal was detected in the areas of the uterus not undergoing decidualization due to the higher abundance of glandular epithelial cells. To assess this further, we measured the relative mRNA levels of all four genes between the NIS and IS tissues on day 7.5 of pregnancy. As shown in Table 6, the mRNA levels of *Hoxb6* and *Prss28* were significantly greater in the NIS tissue than in the IS tissue. On the other hand, the mRNA levels of *Dact1* did not differ significantly between these tissue types. Finally, the mRNA levels of *Nkg7* were significantly greater in the IS tissue than in the NIS tissue.

Discussion

Previous work has compared gene expression in the pregnant uteri to uteri undergoing artificially induced decidualization in an effort to identify genes whose expression levels may be modulated by specific

Figure 4 qRT-PCR analysis of steady-state type 3 aldehyde dehydrogenase mRNA levels in the mouse uterus during decidualization. (A) *Aldh3a1* mRNA levels in bead-induced deciduoma (BID) segment compared with implantation site (IS) segment on day 7.5. (B) *Aldh3a1* mRNA levels on days 3.5–8.5 of pregnancy in the non-implantation site (NIS) segment compared with the IS segment. (C) *Aldh3a2* mRNA levels in the BID segment compared with the IS segment on day 7.5. (D) *Aldh3a2* mRNA levels on days 3.5–8.5 of pregnancy in the NIS segment compared with the IS segment. * $P < 0.05$. Bars represent mean $\pm$ S.E.M. ($N = 4$ –5).

paracrine signals from the conceptus. As a consequence, several genes that are differentially expressed in pregnant mouse uteri during decidualization compared with those undergoing artificially induced decidualization have been identified (recently reviewed in [Herington & Bany \(2009\)](#)). However, these experiments mainly involved the use of either pseudopregnant or ovariectomized hormone-sensitized mice given injections of oil into the uterine lumen to artificially induce decidualization. Recently, we have found that the decidual changes of various artificial models may vary with respect to how they mimic the processes that occur in the pregnant uterus during decidualization ([Herington et al. 2009](#)). The BID model appears to be more 'physiological' than the more commonly used models that use the injection of sesame oil as the deciduogenic stimulus. For example, the pseudopregnant bead-induced model clearly causes the formation of a functional primary decidual zone similar in timing and location to that of pregnant uteri ([Herington et al. 2009](#)). This is not the case for uteri undergoing oil-induced decidualization ([Wang et al. 2004](#), [Herington et al. 2009](#)). More importantly, published data suggest that the progression of decidualization in the BID model is more similar to pregnant uteri than that in oil-induced models ([Herington et al. 2009](#)). Therefore, in this study, a large-scale assessment of the differences in uterine gene expression between the pregnant uterus and those of

pseudopregnant mice undergoing bead-induced decidualization was made. Day 7.5 of pregnancy and pseudopregnancy was the chosen time point since it is well after the process of decidualization begins but is before its completion. Furthermore, prior Affymetrix microarray work comparing gene expression in the day 7.5 decidua compared with oil-induced deciduoma indicated that up to 10% of the genes expressed in the mouse uterus may be impacted by the presence of a conceptus ([Kashiwagi et al. 2007](#)). In contrast, using the pseudopregnant BID model, our work found that $<0.35\%$ of the genes expressed in the mouse uterus during decidualization may be impacted by the presence of a conceptus. We cannot fully explain this discrepancy between our Illumina platform microarray data and the previously published Affymetrix results. A comparison to the Affymetrix microarray data cannot be done since, to our knowledge, the complete dataset of this work has not been made publicly available. Nonetheless, the bead-induced model appears to be more physiologically relevant than the oil-induced deciduoma model ([Herington et al. 2009](#)). It is possible that this is the reason we found significantly fewer differentially expressed genes in this study. However, another possible reason for the lack of identifying a larger number of differentially expressed genes was that our data may have a high false-negative rate. We mainly focus on the set of the genes identified in this study whose expression levels and localization in the uterus may be controlled by signals from the conceptus. However, expression levels of other genes were also studied to verify the lack of differential expression.

Retinoic acid plays a key role in mammalian reproduction and development (reviewed in [Clagett-Dame & DeLuca \(2002\)](#)) but since vertebrates cannot synthesize vitamin A (retinol or retinal) *de novo*, there is a dependence on a source of key dietary provitamin A carotenoids for the production of retinoic acid and related molecules (reviewed in [von Lintig et al. \(2005\)](#)). Depending on the vitamin A levels and timing of the diets, different negative reproductive outcomes occur in rats at different stages of pregnancy ([Clagett-Dame & DeLuca 2002](#)). Although it is assumed that most vitamin A is produced from dietary provitamin A carotenoids within the intestines of mammals ([Olson 1961](#), [Fidge et al. 1969](#), [Duszka et al. 1996](#), [During et al. 1998](#)), it has been noted that the major provitamin A form called β -carotene ([Heinonen 1991](#), [Granado et al. 1996](#), [Strobel et al. 2007](#)) may be stored in various tissues including the uterus and ovary. β -Carotene found in the uterus may have direct antioxidative effects or it may be converted into vitamin A. As it is mostly responsible for cleaving β -carotene to retinal in mice ([Fierce et al. 2008](#)), uterine BCMO1 may be responsible for directing locally stored β -carotene toward retinoic acid at the maternal–conceptus interface. In this study, the expression of the *Bcmo1* gene appears to increase solely

Figure 5 Localization of *Aldh3a1* mRNA in implantation site (IS) and bead-induced deciduoma (BID) segments of pregnant and pseudopregnant mice. Photomicrographs of day 6.5 IS (A and B) or BID (C and D), day 7.5 IS (E and F) or BID (G and H), and day 8.5 IS (I and J) or BID (K and L) segments are shown. AM, anti-mesometrial side; C, conceptus; MM, mesometrial side. Numbers above scale bars are in microns. Representative of at least three independent samples. Global linear adjustments of the brightness and color level were made on the photomicrographs to more accurately represent what was observed on the slides under the microscope.

in the implantation segments of the uterus. Furthermore, we confirmed previous work (Paik *et al.* 2001) that *Bcmo1* is expressed in the maternal tissue near the implanting conceptus undergoing decidualization on days 7.5 and 8.5 of pregnancy. Finally, we found differences in the levels and/or localization of this expression in the BID tissue. This suggests the conceptus may somehow influence *Bcmo1* expression in the endometrium during decidualization. Notably, *Bcmo1* expression is under the control of peroxisome proliferator activator receptors (PPARs) in other tissues (Boulanger *et al.* 2003). PPARs play a role in early pregnancy and placentation (reviewed in Lim & Dey (2002) and Huang (2008)), and previous results showed that *Ppard* expression in the rodent uterus occurs in a region mesometrially to the implanting conceptus (Ding *et al.* 2003) which is similar to what we show for *Bcmo1*. More work is required to determine whether PPARD is responsible for driving *Bcmo1* expression in the uterus during implantation.

Previous work has revealed that several genes involved in retinoic acid metabolism and action are expressed in rodent uteri and developing placenta during implantation (Sapin *et al.* 1997, Zheng & Ong 1998, Vermot *et al.* 2000, Zheng *et al.* 2000) or decidualization, many of which appear to be controlled by progesterone (Jeong *et al.* 2005). Retinoic acid might play a role in implantation, placentation, and decidualization since trophoblast and endometrial cells exhibit the expression of retinoic acid-inducible genes during implantation (Sapin *et al.* 2000).

For example, Yan *et al.* (2001) had shown that retinoic acid promotes the differentiation of trophoblast stem cells to giant cells. Prolactin-like protein expression in the developing placenta may also be influenced by retinoic acid (Lu *et al.* 1994). Finally, retinoic acid can suppress endometrial stromal cell decidualization (Brar *et al.* 1996). *Bcmo1* expression in the mouse uterus during implantation shown in this study suggests that local retinal production from locally stored β -carotene may play a role in implantation. However, since *Bcmo1* knockout mice appear fertile (Lindshield *et al.* 2008), it is doubtful that the local conversion of β -carotene to retinal by decidual cell BCMO1 is absolutely required for mice to have offspring.

Lipid peroxidation at the fetal–maternal interface is likely counterbalanced by maternal protective measures to have an uncomplicated pregnancy. Lipid peroxidation products increase in patients that have complications such as gestational diabetes and preeclampsia during the late phases of pregnancy (Little & Gladen 1999). Even during normal pregnancy, there is an increase in circulating lipid peroxides, especially in the second trimester (Little & Gladen 1999). Since harmful effects of molecules produced by lipid peroxidation can be serious, it is of considerable interest to understand the mechanisms that limit the effects of lipid peroxides generated at the fetal–maternal interface. In this study, we show that *Aldh3a1* expression increases in the maternal tissue surrounding the implanting mouse conceptus in the days leading up to mid-pregnancy.

Figure 6 *Inhibin* and *Fst* mRNA in the mouse uterus during decidualization. (A) *Inhibin* mRNA levels in bead-induced decidualoma (BID) segment compared with implantation site (IS) segments on day 7.5. (B) *Inhibin* mRNA levels on days 3.5–8.5 of pregnancy in the non-implantation site (NIS) segment compared with the IS segment. Localization of *Inhibin* mRNA in the IS and BID tissues of day 7.5 pregnant (C and D) and pseudopregnant (E and F) mice uteri during decidualization on day 7.5. Numbers above scale bars are in microns. *In situ* hybridization is representative of at least three independent samples. Global linear adjustments of the brightness and color level were made on the photomicrographs to more accurately represent what was observed on the slides under the microscope. (G) *Fst* mRNA levels in the BID segment compared with the IS segment on day 7.5. (H) *Fst* mRNA levels on days 3.5–8.5 of pregnancy in the NIS segment compared with the IS segment. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$. Bars represent mean $\pm$ S.E.M. ($N = 4-5$). AM, anti-mesometrial side; C, conceptus; MM, mesometrial side.

The levels and/or localization of this expression, but not that of the related gene *Aldh3a2*, in the endometrium during decidualization appeared to be partially dependent on the presence of a conceptus. ALDH3A1 is an aldehyde dehydrogenase that can break down many of the aldehyde products produced by lipid peroxidation (Townsend *et al.* 2001). Therefore, we speculate the maternal expression of *Aldh3a1* observed in this study may be important to prevent the potential harmful effects of lipid peroxidation products that may be generated at the fetal–maternal interface. More work will be required to determine the precise function of *Aldh3a1* expression in the uterus during pregnancy.

Inhibin expression in the uterus during implantation may differ between species that are considered to exhibit decidualization. The role of inhibins, activins, and follistatin in the uterus during implantation and decidualization has been extensively reviewed elsewhere (Ethier & Findlay 2001, Jones *et al.* 2002, 2006). Briefly,

INHBB may form homodimers to form activin B or may form heterodimers with INHBA or INHA to form activin AB or inhibin respectively. *Inhibin* gene expression in the human uterus appears to be associated with the onset of decidualization. Expression is found in stromal cells undergoing decidualization as well as in immune and glandular epithelial cells with the levels of expression being significantly increased in the stroma of the first semester decidua (Jones *et al.* 2000). Furthermore, the expression of *Inhibin*, but not the related genes *Inhba* or *Inha*, significantly increases in human endometrial stromal cells during decidualization *in vitro* (Horne *et al.* 2008). Several lines of evidence suggested that *Inhibin* is not expressed in the rat endometrium during decidualization (Tessier *et al.* 2003), but rather *Inha* expression is transiently increased in the primary decidual zone (Gu *et al.* 1995). Unlike the rat uterus, this study shows that *Inhibin* expression increases in the mouse uterus in areas undergoing decidualization.

Figure 7 qRT-PCR analysis of *Guca2b* and *Guca2a* mRNA levels in the mouse uterus during decidualization. (A) *Guca2b* and (B) *Guca2a* mRNA levels in the bead-induced deciduoma (BID) segment compared with the implantation site (IS) segment on day 7.5. (C) *Guca2b* mRNA levels on days 3.5–8.5 of pregnancy in the non-implantation site (NIS) segment compared with the IS segment. (D) *Guca2b* mRNA levels in the BID segment compared with the IS segment on days 3.5–8.5 of pseudopregnancy or pregnancy respectively. Bars represent mean $\pm$ S.E.M. ($N=4$). * $P<0.05$, ** $P<0.01$, and *** $P<0.005$.

Furthermore, the observation that *Inhbb* mRNA levels were higher in the BID relative to implantation segments of the uterus leads to the speculation that the presence of a conceptus somehow reduces the increased expression of endometrial *Inhbb* that accompanies decidualization. Notably, reduced uterine *Inhbb* expression and decidualization is observed in women with ectopic pregnancies suggesting that expression may be dependent on decidualization in humans (Horne *et al.* 2008). However, this is unlikely to explain the differences in

expression between the IS and BID tissues observed in this study, as previous work has shown that the degree of decidualization is similar between these tissues (Herington *et al.* 2009). One *in situ* hybridization study described the lack of *Inhbb* expression in the mouse uterus during implantation (Manova *et al.* 1992). However, in a second study, *Inhbb* expression was localized to decidual cells adjacent to the uterine lumen away from the position of the mouse embryo (Feijen *et al.* 1994). Therefore, our results localizing *Inhbb* mRNA to endothelial cells of the vascular islands in the mesometrial decidua and deciduoma are novel. We cannot explain with certainty why discrepancies exist between these three studies. However, expression of *Inhbb* in vascular endothelial cells has previously been detected (Viemann *et al.* 2006, Sha *et al.* 2007, Sobrino *et al.* 2009). Further work is required to better understand *Inhbb* expression and function in the mouse uterus during decidualization.

Guca2a and *Guca2b* genes encode proteins, GUCA2A (guanylin) and GUCA2B (uroguanylin), that bind to a membrane protein called GUCY2C (Sindic & Schlatter 2006a). GUCY2C is a membrane guanylate cyclase, and upon the binding of GUCA2A or GUCA2B there is an increase in cGMP levels within the cells. Recent work has shown that the expression of *Guca2b* in the rat uterus increases during pregnancy then dramatically decreases just prior to parturition (Girotti & Zingg 2003). In this study, we found that *Guca2b* expression is dramatically increased throughout the mouse uterus during decidualization and is differentially expressed between the IS and BID segment tissues. The reason for this differential expression as well as the function of the *Guca2b* expression in the uterus during rodent pregnancy is not completely understood. It has been speculated that GUCA2B may act on the myometrium and cause myometrial quiescence during pregnancy by increasing cGMP levels (Girotti & Zingg 2003). However, to the best of our knowledge, the expression of *Gucy2c* in the rodent myometrium has not been described in the literature, and we were not successful in detecting expression in the mouse uterus during decidualization. Regardless, a *Guca2b* DNA vaccine has been shown to have anti-fertility effects in mice (Sun *et al.* 2008). It remains to be determined whether

Table 4 qRT-PCR to assess mRNA levels between the day 7.5 implantation (IS) segment tissues containing *Hand1* wild-type (WT) and knockout (KO) conceptuses.

Symbol	WT	KO	P value
<i>Aldh3a1</i>	1.55 $\pm$ 0.56	2.35 $\pm$ 0.90	0.251
<i>Bcmo1</i>	1.00 $\pm$ 0.11	1.38 $\pm$ 0.56	0.48
<i>Guca2b</i>	1.00 $\pm$ 0.30	1.30 $\pm$ 0.75	0.56
<i>Inhbb</i>	1.00 $\pm$ 0.12	0.78 $\pm$ 0.05	0.36

Values represent the mean ($\pm$ S.E.M.; $N=5$) relative mRNA levels normalized to *18S* rRNA levels.

Table 5 qRT-PCR to assess mRNA levels between the day 7.5 implantation (IS) and bead-induced decidualoma (BID) segment tissues of pregnant and pseudopregnant mice respectively.

Symbol	BID	IS	P value
<i>Dact1</i>	1.5 ± 0.30	1.0 ± 0.25	>0.05
<i>Hoxb6</i>	2.6 ± 0.83	1.0 ± 0.30	>0.05
<i>Nkg7</i>	1.7 ± 0.88	1.0 ± 0.23	>0.05
<i>Prss28</i>	1.8 ± 0.40	1.0 ± 0.13	>0.05

Values represent the mean ($\pm$ s.e.m.; $N=4$) relative mRNA levels normalized to 18S rRNA levels.

the anti-fertility effect is specifically due to altered uterine GUCA2B action. Finally, if *Gucy2c* is not expressed in the uterus, it seems plausible that GUCA2B may function only as an endocrine factor produced in the uterus during pregnancy. Alternatively, if *Gucy2c* is expressed in the conceptus, uterine GUCA2B might be a paracrine factor and exert effects on the conceptus. Future studies interrogating the function of *Guca2b* expression in the uterus during the peri-implantation period are warranted.

Paracrine signals from trophoblast cells are believed to control the expression of endometrial gene expression in several ungulate species and humans, but evidence for this is less obvious for rodents (reviewed in [Herington & Bany \(2009\)](#)). Since trophoblast giant cells are the part of the conceptus in most intimate contact to the maternal tissue, we have previously hypothesized that these cells may secrete paracrine factors that regulate uterine gene expression during decidualization ([Bany & Cross 2006](#)). However, it seems that signals from the trophoblast giant cells are not required for the differential expression of *Aldh3a1*, *Bcmo1*, *Inhbb*, and *Guca2b* observed in this study, as expression levels are not different between implantation segments containing *Hand1* knockout compared with wild-type conceptuses. In fact, we have not been able to identify differential expression between such samples by microarray analysis (BM Bany, unpublished data). This suggests that up to day 7.5 of pregnancy, the giant cells may have very little effect on endometrial gene expression during decidualization in the mouse. We speculate that paracrine factors from other components of the conceptus may be responsible for the differential expression observed in this study. Alternatively, the difference between the physical stimuli of a growing conceptus to that of a fixed blastocyst-sized bead may be responsible. Furthermore, whether giant cells exert paracrine effects on the endometrium during later days of pregnancy as the major phases of placental development occur still needs to be determined.

In conclusion, we have demonstrated differential gene expression in the uterus during decidualization in pregnant mice compared with bead-induced pseudo-pregnant mice. This differential gene expression supports a previous suggestion that signals from the rodent

conceptus may influence uterine gene expression during decidualization ([Herington et al. 2009](#)). However, the much lower magnitude of the differential expression observed in this study is in contrast to previous microarray work ([Kashiwagi et al. 2007](#)), where the oil-induced decidualoma model was used. These differences may be explained, at least in part, to recent observations that the BID model more closely mimics pregnancy than the other commonly used decidualoma models ([Herington et al. 2009](#)). Notably, this study provides the identity of several genes whose expression in the uterus during decidualization may be influenced by molecular or physical signals from the conceptus.

Materials and Methods

Animals and tissue collection

All procedures involving mice were approved by Southern Illinois University Institutional Animal Care and Use Committee. Female (9–12 weeks old) and male (3–10 months old) CD1 mice (Charles Rivers Laboratory, Wilmington, MA, USA) were housed under controlled light conditions (lights on: 0700 to 1900 h) and allowed free access to food (Formulab 5008 LabDiet; Purina Mills, Gray Summit, MO, USA) and water. Females were mated with fertile males, and the morning a vaginal plug was observed was termed day 0.5

Figure 8 Localization of *Prss28* mRNA in the mouse uterus on day 7.5 of pregnancy or pseudopregnancy. Representative sections from (A) non-implantation (NIS) and (B and C) implantation site (IS) segments from pregnant uteri. Representative sections from (D) non-bead (NB) and (E and F) bead-induced decidualoma (BID) segments of pseudo-pregnant uteri. AM, anti-mesometrial side; B, bead stimulus; C, conceptus; MM, mesometrial side. Numbers above scale bars are in microns. Sections are representative of at least three independent samples. Global linear adjustments of the brightness and color level were made on the photomicrographs to more accurately represent what was observed on the slides under the microscope.

Table 6 qRT-PCR to assess mRNA levels between the day 7.5 non-implantation (NIS) and implantation (IS) segment tissues of pregnant mice.

Symbol	NIS	IS	P value
<i>Dact1</i>	1.9±0.42	1.0±0.15	>0.05
<i>Hoxb6</i>	17.8±4.20	1.0±0.13	<0.005
<i>Nkg7</i>	0.3±0.02	1.0±0.15	<0.005
<i>Prss28</i>	9.7±2.74	1.0±0.37	<0.001

Values represent the mean ($\pm$ S.E.M.; $N=4$) relative mRNA levels normalized to 18S rRNA levels.

of pregnancy. The model used to obtain artificially induced decidualomas for this study has been described in detail elsewhere (Herington *et al.* 2009). Briefly, female CD1 mice were mated with vasectomized males, and the morning a vaginal plug was observed was considered day 0.5 of pseudopregnancy. On day 2.5, between 1300 and 1500 h, blastocyst-sized concanavalin A-coated agarose beads (Sigma) were transferred into the uterine lumen. This BID model was chosen over other artificial decidualization models, because it better recapitulates the decidual changes that occur in the pregnant uterus compared with other commonly used models (Herington *et al.* 2009).

Animals were killed at 0900 h on days 3.5–8.5 of pregnancy or pseudopregnancy for tissue collection. The uterine horns were dissected and cleared of mesentery. In the case of uteri collected on day 4.5 of pregnancy or pseudopregnancy, implantation and bead-containing segments of the uterus were visualized by an i.v. injection of Evans blue dye as described previously (Herington *et al.* 2009). For days 4.5–8.5 uterine horns, the IS or BID segments were separated from NIS or NB segments respectively. For implantation segments, all tissues of the conceptus (embryo and trophoblast cells) were carefully dissected out and discarded. All the remaining tissue were then pooled and considered IS segment tissue.

To assess the potential function of giant cells on conceptus-dependent gene expression levels in the uterus, *Hand1* mutant mice were used. *Hand1*-deficient conceptuses fail to develop past days 7.5–9 of pregnancy, and the failure of the conceptus appears to be due to lack of trophoblast giant cells (Riley *et al.* 1998). Heterozygous animals were intercrossed to obtain timed pregnant female mice carrying wild-type, heterozygous, and knockout conceptuses and used as described previously (Bany & Cross 2006). PCR genotyping of the embryos was carried out by PCR as described by Riley *et al.* (1998).

RNA extraction

Tissues were homogenized in TRIzol reagent, and total RNA was extracted as recommended by the manufacturer (Invitrogen). The total RNA was treated with Turbo DNase as recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA) and was then re-extracted with TRIzol reagent. Total RNA concentrations were measured spectrophotometrically, and samples were subjected to denaturing agarose gel electrophoresis to verify RNA integrity and purity.

Illumina microarray analysis

This study was to extend the findings of previous work (Bany & Cross 2006, Kashiwagi *et al.* 2007) that directly compared the development and function of decidua and decidualoma at 3 days after the onset of decidualization. Therefore, RNA isolated from the uterine tissue from day 7.5 IS and BID segments were used for the microarray analysis ($N=3$). The RNA quality was further verified using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) before microarray analysis. The total RNA was then used for microarray analyses using the Illumina platform and a whole-genome Expression BeadChip array slide (MouseWG-6 v1.1; Illumina, Inc., San Diego, CA, USA) to determine differences in steady-state mRNA levels between the tissues. The technology used in this platform involves microbeads coated with oligonucleotide probes and is described in detail elsewhere (Fan *et al.* 2006). Briefly, each MouseWG-6 v1.1 slide contains six separate whole-genome gene expression arrays, allowing six target samples to be hybridized to six independent arrays on one slide. Each array contains over 46 000 different oligonucleotide probes. Target preparation, hybridization, and scanning were conducted by the Microarray Facility at the Hamilton Eye Center (UT Memphis) using the exact methods suggested by the manufacturer (Illumina, Inc.). Briefly, total RNA isolated from the tissue for each experiment was used to create six biotin-labeled cRNA target samples ($N=3$) using the Illumina RNA amplification kit (Applied Biosystems). 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 BeadArray Reader with BeadStudio software (Illumina, Inc.).

Data analysis

Complete microarray data can be found at NCBI's GEO database (GSE25903, www.ncbi.nlm.nih.gov/geo). Raw intensity data were background subtracted and 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 (Chipster.csc.fi). Chipster, developed by the Finnish IT Center for Science CSC (Chipster.csc.fi), is a useful graphical user interface to a collection of online microarray analysis tools such as R/Bioconductor (Gentleman *et al.* 2004). The scale normalization (Smyth & Speed 2003) tool of Chipster, which uses the limma R package, was used to normalize the data. Significance of differential expression was determined using two different methods. First, the Chipster tool that uses the Bioconductor package limma Empirical Bayes method (Smyth 2004) using the default Benjamini–Hochberg method to control the false discovery rate was used. Differences were considered significant if the adjusted P values were <0.05 . In the second analysis, the Reproducibility-Optimized Test Statistic (ROTS) tool was used with a cutoff false discovery rate of <0.05 . The ROTS procedure enables the selection of a suitable gene ranking statistic directly from the given dataset and is described in detail elsewhere (Elo *et al.* 2008, 2009). The lists of differentially expressed genes were combined and annotated using Bioconductor annaffy package. The hypogeometric test

for GO tool of Chipster was used to determine over- and underrepresented go terms of the list of differentially expressed genes using a cutoff of $P < 0.01$ for statistical significance. Finally, a heat map was constructed using the clustering tool of Chipster to visually show signal levels detected for each sample of the differentially expressed genes.

Quantitative real-time PCR

qRT-PCR was carried out as described previously (Herington *et al.* 2009) using oligonucleotide primers (IDT Technologies, Coralville, IA, USA) shown in [Supplementary Table 3A](#). 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 (Herington & Bany 2007a). The efficiencies of the PCR were linear between the mRNAs and 18S rRNA and were >85%. Differences between mean levels of the steady-state mRNA levels were determined statistically as described previously (Herington & Bany 2007a).

In situ hybridization

To collect tissue for *in situ* hybridization, mice were anesthetized with an i.p. injection of a ketamine hydrochloride (200 mg/kg) and xylazine (20 mg/kg; Henry Schein, Melville, NY, USA). Perfusion of PBS and fixative were then performed as described previously (Herington & Bany 2007b). Uteri were then dissected out, and uterine samples were immersed in fresh 4% (w/v) paraformaldehyde in PBS for 24 h, then in 70% (v/v) ethanol for 24 h at 4 °C. Processing of tissue into paraffin was done using routine methods and then 5 µm sections were mounted onto HistoBond glass slides (Statlab, McKinney, TX, USA).

To prepare templates for sense and antisense riboprobe synthesis, PCR amplicons were generated using TopTaq DNA polymerase and purified using QIAquick PCR purification kits as recommended by the manufacturer (Qiagen) using the primers shown in [Supplementary Table 3B](#). The amplicons were then cloned into the pGEM-T Easy vector (Promega), which contains SP6 and T7 RNA polymerase sites flanking the multiple cloning region. After sequencing the cDNA clones, the plasmids were used to generate transcription templates by PCR using TopTaq (Qiagen) and M13 forward or reverse primers plus the appropriate insert-specific primer. 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 MAXIscript SP6 kits (Applied Biosystems) along with dioxigenin-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 (data not shown).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-10-0358>.

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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