Progesterone-induced RNA *Hand2os1* regulates decidualization in mice uteri

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Abstract

Decidualization is a critical process for successful embryo implantation and subsequent placenta formation. The characterization and physiological function of lncRNA during decidualization remain largely unknown. In the present study, we conducted RNA-sequencing analysis to compare gene expression between decidua of days 6 and 8, and normal pregnant endometrium (day 4). A total of 2332 high-confidence putative lncRNA transcripts were expressed. Functional clustering analysis of *cis* and *trans* lncRNA targets showed that differentially expressed lncRNAs may regulate multiple gene ontology terms and pathways that have important functions in decidualization. Subsequent analyses using qRT-PCR validated that eight of all lncRNAs were differentially regulated in mice uteri during decidualization, both *in vivo* and *in vitro*. Furthermore, we showed that differentially expressed lncRNA of *Hand2os1* was specifically detected in stromal cells on days 2 to 5 of pregnancy and was strongly upregulated in decidual cells on days 6–8 of pregnancy. Similarly, *Hand2os1* expression was also strongly expressed in decidualized cells following artificial decidualization, both *in vivo* and *in vitro*. In uterine stromal cells, P₄ was able to significantly upregulate the expression of *Hand2os1*, but upregulation was impeded by RU486, whereas E₂ appeared to have no regulating effect on *Hand2os1* expression. Concurrently, *Hand2os1* significantly promoted the decidual process *in vitro* and dramatically increased decidualization markers *Prl8a2* and *Prl3c1*. Our results provide a valuable catalog for better understanding of the functional roles of lncRNAs in pregnant mouse uteri, as it relates to decidualization.

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Introduction

In early pregnancy, the extensive remodeling of maternal endometrium is critical for successful implantation of the blastocyst and subsequent placenta formation, which is characterized by rapid proliferation and differentiation of endometrial stromal cells (ESCs) into decidual cells. This process is known as decidualization. The morphological and functional transformation of this process in decidual stromal cells contributes to uterine hemostasis, angiogenesis, and immune response during implantation and pregnancy (Schatz *et al.* 2016). Defective decidualization at the site of implantation is associated with several pregnancy complications, such as early pregnancy defects/loss, preeclampsia, utero-placental disorders, endometriosis, intrauterine growth restriction, and recurrent pregnancy loss (Mori *et al.* 2016, Garrido-Gomez *et al.* 2017). On day 4 of mouse pregnancy (day 1 = vaginal plug), ovarian P₄ and pre-implantation E₂ secretion renders the uterus receptive to the blastocyst, which is the prerequisite for embryo implantation and decidualization. The proliferating

stromal cells at the antimesometrial pole surrounding the implanting blastocyst undergo differentiation into decidual cells and form the primary decidual zone (PDZ) on roughly the afternoon of day 5. During days 6–7 of pregnancy, stromal cells adjacent to the PDZ continue to proliferate and differentiate into polyploidy decidual cells, forming the secondary decidual zone (SDZ). At this point, the cells comprising the PDZ have degenerated progressively by apoptosis (occurring on day 7 of pregnancy) and have for the most part disappeared at day 8. After day 8, placental and embryonic growth slowly replaces the SDZ (Das 2009, 2010). Numerous signaling molecules and factors, including homeobox transcription factors, cell-cycle molecules, cytokines, growth factors, signaling mediators, extracellular matrix remodeling factors, steroid hormones, and endometrial autocrine and paracrine factors, have been implicated in the progression of decidualization. Nevertheless, the precise cellular and molecular mechanism underlying decidualization remains unclear.

Transcriptome analysis revealed that more than 90% of DNA sequences actively transcribed proteins but that

only 2% of it is responsible for encoding proteins. Thus, the majority of transcripts are referred to as ncRNAs, which can be categorized into infrastructural ncRNAs and regulatory ncRNAs (Birney *et al.* 2007). On the basis of their size, regulatory ncRNAs can be classified into small ncRNAs and lncRNAs (Ponting *et al.* 2009). Small ncRNAs, such as miRNA, have been found to play essential roles in endometrial receptivity, decidualization, and embryo implantation (Liang *et al.* 2017, Paul *et al.* 2019). lncRNAs are transcripts longer than 200 nucleotides that are frequently capped, polyadenylated, and spliced, with highly tissue-specific/cell-specific expression patterns and less conservation across multiple species. Recent studies revealed that lncRNAs are associated with oogenesis, ovary development, oocyte maturation, and embryo development, trophoblast cell migration, as well as placenta, suggesting that lncRNAs may play an important functional role in female mammalian reproductive processes (Hamazaki *et al.* 2015, Taylor *et al.* 2015, Bouckenheimer *et al.* 2016, Liu *et al.* 2018). In contrast, the expression and biological function of lncRNAs during embryo implantation remain largely unknown.

In this paper, we report systematic identification of lncRNAs in the endometrial tissue of pregnant mice on day 4 (D4U), day 6 (D6U), and day 8 (D8U) at implantation sites, using RNA sequencing (RNA-seq). Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were carried out to predict the possible functions of *cis* and *trans* lncRNA targets. Eight differentially expressed lncRNAs were validated by quantitative RT-PCR (qRT-PCR) during *in vivo* and *in vitro* decidualization. One lncRNA *Hand2os1* (*Uph*, also known as *HAND2-AS1* in humans) was selected for further investigation of the biological functions involved in the decidualization process, using over-expressing and silencing. Our results suggest that P_4 induced *Hand2os1* expression in ESCs in a progesterone receptor (PR)-dependent manner; this process may in turn be involved in the decidual process, via regulation of the following decidualization markers: prolactin family 8, subfamily a, member 2 (*Pr18a2*) and prolactin family 3, subfamily c, and member 1 (*Pr13c1*) expression. The present study serves as a valuable resource for the systematic annotation and expression profiling of lncRNAs and provides a foundation for future studies aimed at understanding their role in decidualization.

Materials and methods

Ethics statement

The methods used in this study were performed in accordance with the guidelines of the Committee for the Ethics on Animal Care and Experiments at Northwest A&F University. All experimental protocols involving animal subjects were

approved by the Committee for the Ethics on Animal Care and Experiments at Northwest A&F University.

Animals, treatments, and sample collection

Mature mice (Kunming white strain) aged 8–10 weeks were purchased from the Laboratory Animal Center of The Fourth Military Medical University (Shaanxi, China). The mice were caged in a temperature and light-controlled room (12 h light:12 h darkness cycle). Food and water were provided *ad libitum*.

Female mice were mated with fertile male mice from the same breed to induce pregnancy. Pregnancy on days 1 and 4 (day 1 = day of vaginal plug) was confirmed by recovering embryos from the oviduct and uterus, respectively. On the above pregnant days, mice were killed at 09:00 h by cervical dislocation, and the entire endometrium and myometrium were removed by extrusion method and immediately collected. Decidual tissue was collected from the implantation site on days 6 to 8 of pregnancy; the procedure was performed according to that described by Shea and Geijsen (2007). All samples were immediately transferred into an RNAstore Reagent (Tiangen, Beijing, China) and stored at -20°C . The endometrium of five mice during each pregnant day was pooled together for further RNA analysis and library construction. Three biological replicates per sample, combined from five mice, were performed.

Mouse models involving pseudopregnancy, artificial decidualization, and hormonal treatment were produced as described in our previous reports (Lan *et al.* 2013, Lin *et al.* 2014, Xiong *et al.* 2016). Pseudopregnant female mice were obtained by mating to vasectomized males of proven sterility, and the entire uterus of pseudopregnant mice was collected between days 1 and 6. One of the uterine horns were infused with 25 μL sesame oil on day 4 of pseudopregnancy to induce artificial decidualization, and the contralateral, untreated horn served as a control. In the hormone treatment model, the ovariectomized mice were subcutaneously injected after 2 weeks with either E_2 (100 ng/mouse), P_4 (1 mg/mouse), a combination of both, or P_4 (1 mg/mouse) plus RU486 (10 mg/mouse) (Sigma). Following on, uterine tissue was collected 24 h following the previously mentioned treatment. Meanwhile, the control animals received the sesame amount of sesame oil (0.1 mL/mouse).

Isolation of mouse ESCs and induction of decidualization in vitro

Uterine horns of day 4 pregnant mice were dissected longitudinally, washed with Hanks' balanced salt solution (HBSS), and cut into 3–5 mm pieces. Each uterine horn was placed in HBSS containing 0.25% trypsin (Sigma) for 1 h at 4°C , followed by 1 h at room temperature, and then 10 min at 37°C . After discarding the supernatant and washing twice with HBSS, the remaining tissue was then incubated in HBSS containing 0.05% collagenase (Sigma) at 37°C for 45 min. The digested tissue was vortexed for 20 s and filtered through a 70 μm filter (Cell Strainer, Millipore). The isolated cells were re-suspended and washed with a DMEM/F12 culture medium and then seeded in a DMEM/F12 culture medium with

100 U/mL penicillin, 100 µg/mL streptomycin, and 10% charcoal-treated fetal bovine serum (cFBS, Life Technologies) at a concentration of 5×10^5 cells/well in 60 mm dishes. Following incubation for 2 h, unattached cells were removed by wash with HBSS. The attached cells were plated in DMEM/F12 with 10% cFBS and allowed to further attach for 6 h. Then, stromal cells were treated with 10 nM estradiol-17β (Sigma) and 1 µM P₄ (Sigma) to induce decidualization, and cells were harvested 0, 24, 48, and 72 h later. Mouse *Prl8a2* was detected using qRT-PCR as a reliable decidualization marker.

RNA-seq analysis

LncRNA expression profiles of mouse decidualized endometrium were generated using an Illumina HiSeq™ 2500 platform and analyzed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). The library construction, quality control, transcriptome assembly, and screening of candidate lncRNAs were obtained as Supplementary methods (see section on [supplementary materials](#) given at the end of this article). Paired-end clean reads of high quality were mapped to the mouse GRCh38.p6 reference genome (ftp://ftp.ncbi.nlm.nih.gov/genomes/Mus_musculus/Assembled_chromosomes/seq/). Sub-types of the annotated lncRNAs were included in the latest gene/transcript biotypes in the Ensembl database. We searched coding genes 100k upstream and downstream of all identified lncRNAs to represent putative *cis* target genes. For *trans* fashion, we calculated the expressed correlation between lncRNAs and coding genes, using custom scripts to search common expression modules, and subsequently analyzed their function. The differentially expressed lncRNAs between any two samples were identified using a *P*-adjust <0.05, according to fragments per kilobase of exon per million fragments mapped (FPKM). The GO initiative and the KEGG database were used to conduct functional enrichment analysis of the targeted genes of differentially expressed lncRNAs. The lower the *P* value, the more prominent the relevance; only GO terms or KEGG pathways with corrected *P*-values <0.05 were considered as being significantly enriched.

qRT-PCR analysis

Total RNA was extracted using TRIzol Reagent (Invitrogen). RT was performed using the 5X All-In-One RT MasterMix with AccuRT Genomic DNA Remove Kit (Applied Biological Materials Inc. BC, Canada), as per the manufacturer's instructions. qRT-PCR was performed using the EvaGreen qPCR Mastermix Kit (Applied Biological Materials Inc. BC, Canada) on the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). All reactions were conducted in biological triplicate. The relevant primers and temperatures employed are listed in Supplementary Table 1. The authenticity of the PCR products was verified by melting curve analysis and 2% agarose gel electrophoresis. Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ analysis method. Final data were expressed as the mean $\pm$ S.E.M. and analyzed using a one-way ANOVA, followed by Fisher's least significant difference test and independent samples *t*-test, using SPSS software (v.23.0;

SPSS, Inc.). Differences were considered significant when *P* was <0.05.

Detecting target RNA via RNAscope

Uterine tissue were formalin-fixed, paraffin-embedded, and sliced into 5 µm thick sections for detecting target RNA via RNAscope (Advanced Cell Diagnostics, Newark, CA, USA). Detection of *Hand2os1* expression via probe targeting was performed on uterine tissue using an RNAscope 2.5 High Definition-BROWN Assay Kit, as per the manufacturer's instructions (Advanced Cell Diagnostics, USA). Finally, the previously noted tissue sections were counterstained with hematoxylin. Images were acquired using a fluorescence microscope (Nikon).

Transfection of lncRNA siRNA and overexpression vector

Mouse ESCs were transfected with a *Hand2os1* siRNA or negative control, designed, and synthesized by Gene Pharma Co., Ltd. (Shanghai, China), using a TurboFect Transfection Reagent (Thermo Scientific), as per the manufacturer's instructions. Following incubation with the lncRNA siRNA for 24 h, the medium was replaced with DMEM/F12 containing 10% cFBS, 10 nM E₂, and 1 µM P₄ to induce decidualization. The previously noted cells were harvested at the indicated time points for decidual marker gene analysis. The target sequences of siRNAs were as follows: 5'-GGGAACUGACUGCAGUCCUTT and 5'-AGGACUGCAGUCAGUCCCTT (*Hand2os1* siRNA); 5'-UUCUCCGAACGUGUCACGUTT and 5'-ACGUGACACGUUCGGAGAATT (non-specific scrambled siRNA, negative control).

Full-length *Hand2os1* cDNA fragments from mice uteri were amplified by PCR using the following primers: 5'-GAATTC (*EcoRI*) CACTCATAACCATAAGATAATTAACCGG and 5'-TCTAGA (*XbaI*) TTTAAAAA TAATTTTAAATATACTATGTGCATGGTTGG. The *Hand2os1* and pcDNA3.1 vector were cut by *EcoRI*/*XbaI* (Takara) at 37°C for 12 h; the target segments were then ligated into pcDNA3.1 using SolutionI (Takara Biotechnology) at 16°C for 4 h, in order to construct pcDNA3.1-*Hand2os1*. The vector was transfected into mouse ESCs using a TurboFect Transfection Reagent (Thermo Scientific), as per the manufacturer's instructions. An empty pcDNA3.1 vector was used as a control.

Results

Decidual tissue collection and validation

To identify the integrity of isolated endometrium and confirm that the embryo and myometrium had been removed intactly, the mRNA expression of decidualization marker genes, alkaline phosphatase *Alpl* and *Prl8a2*, were examined, respectively. Consistent with previous reports, *Prl8a2* mRNA was significantly up-regulated, which accompanied decidualization (Supplementary Fig. 1A). Additionally, *Alpl*, another

typical decidualization marker, was detected at high levels on day 6 of pregnancy but decreased on day 8 (Supplementary Fig. 1B). This pattern was similar to those previously reported in mice (Pollard *et al.* 1990, Herington *et al.* 2009). Meanwhile, caudal type homeobox 2 (*Cdx2*), the transcription factor expressed specifically in the developing trophoctoderm (Wu *et al.* 2010) was not detected in our isolated endometrium on pregnancy days 4, 6, or 8, respectively. Furthermore, the significantly reduced mRNA levels of a smooth muscle-maker gene, alpha smooth muscle actin (*Acta2*) (Gao *et al.* 2015), was observed in the isolated endometrium, compared with the entire group of uterine samples for the same pregnant days, respectively ($P < 0.01$, Supplementary Fig. 1C). *Acta2* was consistently measured on blood vessels throughout the endometrial stroma during early pregnancy (Kaitu'u-Lino *et al.* 2012).

Genome-wide identification and characterization of lncRNAs in mouse endometrium during decidualization

In order to systematically detect the expression of lncRNAs during decidualization, cDNA libraries were constructed from mouse endometrium on pregnant D4U, D6U, and D8U, respectively. An average of between 104,000,000 and 122,000,000 raw reads were generated, and approximately 97.02–97.12% of clean reads among raw reads in each sample were obtained following stringent filtering (Supplementary Table 2). Subsequently, we mapped the clean reads to the reference genome of *Mus musculus* using TopHat v2.0.9. The percentage of mapped clean reads in each sample ranged from 89.90 to 91.54%, of which 64.04 to 73.20% were mapped uniquely to only one location. The mapped reads from each sample were assembled and merged using Cufflinks (v2.1.1) and Cuffmerge, respectively, and a total of 127,671 unique assembled transcripts were produced.

In order to obtain a comprehensive lncRNA transcript set, highly stringent filtering conditions were implemented, as shown in Supplementary Fig. 2, according to the structural and functional characteristics of lncRNAs. Finally, a total of 2332 putative lncRNA transcripts were identified, including 1822 annotated lncRNA transcripts (1439 genes) and 510 novel lncRNA transcripts (388 genes) (Supplementary Table 3). Overall, the genomic features of novel lncRNAs were similar to the annotated lncRNAs in our study. Consistent with existing reports, differences in genomic features were observed between lncRNAs and protein-coding genes (Supplementary Fig. 3).

Identification of differentially expressed lncRNAs in decidualized cells

Upon further analysis, according to FPKM values, 334 differentially expressed lncRNA transcripts were

identified in mouse endometrium during decidualization (Supplementary Table 4). There were 182 differentially expressed lncRNA transcripts in D6U vs D4U groups, of which 77 transcripts were significantly upregulated and 105 transcripts were significantly downregulated. Furthermore, a total of 226 lncRNA transcripts were differentially expressed, on the basis of pairwise comparisons between the D8U and D4U groups, with 99 transcripts upregulated and 127 transcripts downregulated. We identified 87 lncRNA transcripts with significant expression differences between the D8U and D6U groups among these 44 upregulated and 43 downregulated transcripts. Overall, the number of downregulated lncRNAs was higher than the number of upregulated lncRNAs during decidualization. As shown in Table 1 and Supplementary Table 4, 16 differentially expressed lncRNA transcripts were commonly identified in all three groups. Meanwhile, two lncRNA transcripts (*ENSMUST00000184645.1* and *ENSMUST00000126270.1*) were differentially upregulated and five lncRNA transcripts (*ENSMUST00000172910.1*, *ENSMUST00000181955.1*, *ENSMUST00000186411.1*, *ENSMUST00000185789.1*, and *LNC_000225*) were differentially downregulated, in a stage-dependent manner.

GO and KEGG pathway analysis of differentially expressed lncRNA target genes

To investigate the potential functions of lncRNAs, we first predicted the differentially expressed lncRNA *cis*- and *trans*-acting target genes (Supplementary Tables 5 and 6). The significantly enriched *cis* GO terms of lncRNA targets in the D6U vs D4U groups were similar to D8U vs D4U groups and associated primarily with cell proliferation, differentiation, migration, and angiogenesis, which contributed to endometrium re-building and decidualization (Supplementary Fig. 4). In addition, the top 30 significantly enriched *trans* GO terms were similar in the three comparison groups, indicating that the regulating function of these lncRNAs may be preserved in the process of decidualization. The majority of enriched GO terms of the differential expression lncRNA target genes in the present study were similar to previous work conducted involving uterine mRNA expression profiles during decidualization (McConaha *et al.* 2011, Sroga *et al.* 2012, Liu & Wang 2015).

Compared with those in the D4U group, the most enriched KEGG pathways of *cis* and *trans* target lncRNAs in the D6U and D8U groups primarily included a metabolic pathway, estrogen signaling pathway, cancer pathway, the PI3K-Akt signaling pathway, HTLV-I infection, the MAPK signaling pathway, and the Ras and Ras1 signaling pathways. Meanwhile, the most enriched pathways in the D8U vs D6U groups for *cis* included an oxytocin signaling pathway, a cAMP signaling pathway, and a calcium signaling pathway (Supplementary Tables 7 and 8).

Table 1 Sixteen differentially expressed lncRNA transcripts were commonly identified in all the three groups.

Transcript ID	Gene ID	Gene name	D4U FPKM	D6U FPKM	D8U FPKM
ENSMUST00000136359.3	ENSMUSG00000000031.11	H19	40.5661	131.543	65.4112
LNC_000368	XLOC_032091		0.242036	3.30358	1.47354
ENSMUST00000172910.1	ENSMUSG00000091562.3	Gm9581	18.467	10.0689	5.6804
ENSMUST00000184645.1	ENSMUSG00000062036.8	Gm19518	0	1.36506	6.52689
ENSMUST00000151426.1	ENSMUSG00000085114.1	1600025M17Rik	0	13.0193	0.584374
LNC_000021	XLOC_003243		2.53321	0.0612518	0.464945
ENSMUST00000181955.1	ENSMUSG00000074415.6	2610203C20Rik	6.46048	1.5733	0.321162
ENSMUST00000181075.1	ENSMUSG00000096980.1	Gm26526	1.07335	3.43487	0.116526
ENSMUST00000156068.2	ENSMUSG00000018451.6	6330403K07Rik	11.1774	0.749637	19.8742
ENSMUST00000126270.1	ENSMUSG00000085931.1	Gm12648	0	2.19169	5.76829
LNC_000225	XLOC_019711		15.6081	2.52168	0
ENSMUST00000186386.2	ENSMUSG00000100510.2	Hand2os1	36.048	276.613	97.4685
ENSMUST00000186411.1	ENSMUSG00000100303.1	2600014E21Rik	1.33655	0.55117	0.20727
ENSMUST00000192860.1	ENSMUSG00000104022.1	CAAA01187608.2	0.890325	16.9395	3.18089
ENSMUST00000185789.1	ENSMUSG00000101609.1	Kcnq1ot1	6.38982	4.04542	2.66331
ENSMUST00000128111.1	ENSMUSG00000087067.1	Gm11532	0.096742	2.1489	0.821279

Experimental validation of differentially expressed lncRNAs

To validate RNA-seq data, we selected eight differentially expressed lncRNAs and examined their expression patterns during the decidualization process by qRT-PCR

(Fig. 1). In general, the qRT-PCR results confirmed that the expression patterns of these lncRNAs were consistent with their expression trend, calculated from the RNA-seq data. Compared with those at day 1 of pregnancy, the RIKEN cDNA C630043F03 gene (*C630043F03Rik*) and predicted gene 14133 (*Gm14133*) were significantly

Figure 1 Validation of eight differentially expressed lncRNAs by qRT-PCR in pregnant mice uteri. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene for normalization. The data are expressed as the mean $\pm$ S.E.M. of three replicates, and the bar bearing different superscripts indicates a significant difference between the mean values ($P < 0.05$).

Figure 2 The qRT-PCR analyses of expression levels for differentially expressed lncRNAs during decidualization *in vitro*. The mouse ESCs collected on day 4 of pregnancy were decidualization induced with P_4 (1 μ M) and E_2 (10 nM). Then, the ESCs were harvested at various times for qRT-PCR analysis. GAPDH was used as a reference gene for normalization. *Prl8a2* was detected using qRT-PCR as a reliable decidualization marker. The data are expressed as the mean $\pm$ S.E.M. of three replicates, and the bar bearing different superscripts indicates a significant difference between the mean values ($P < 0.05$).

upregulated at day 4 of pregnancy, and no significant difference was observed on days 6–8 of pregnancy ($P < 0.05$). Deiodinase, iodothyronine type III, opposite strand upstream RNA (*Dio3os*), predicted gene 12840 (*Gm12840*), and ganglioside-induced differentiation-associated protein 10 (*Gdap10*) expression levels from days 6–8 of pregnancy were significantly higher than those for days 1 and 4, respectively ($P < 0.05$). Interestingly, MAP/microtubule affinity-regulating kinase 1 and pseudogene 3 (*Gm19518*) was not observed for days 1 and 4 pregnant mouse uteri, and the expression level of *Gm19518* was significantly upregulated during decidualization. The highest expression level was detected on day 8 of pregnancy ($P < 0.05$). Compared with those on days 1 and 4 of pregnancy, *LNC000368* and *Hand2os1* expression levels increased dramatically on day 6 and were then significantly down-regulated on day 8 of pregnancy ($P < 0.05$).

To evaluate the physiological function of the previously validated lncRNAs, which obtained significant up- or downregulation during decidualization *in vivo*, we further detected its expression changes during decidualization *in vitro* and under artificial decidualization, respectively (Figs 2 and 3). In agreement with the results of RNA-seq data, no significant difference was detected in *C630043F03Rik* level during decidualization *in vitro*. *Dio3os*, *Gm12840*, *Gdap10*,

and *LNC000368* levels significantly increased in a time-dependent manner during ESC decidualization *in vitro* ($P < 0.05$). Furthermore, *Gm14133* and *Gm19518* were not detected in either cultured ESCs or decidual ESCs *in vitro*. Consistent with the results, the expression levels of *Dio3os*, *Gm12840*, *Gdap10*, *LNC000368*, and *Hand2os1* were also significantly higher in the group undergoing artificial decidualization, compared with those in the control group ($P < 0.05$). Taken together, these results further support the reliability of RNA-seq data and indicate that these differentially expressed lncRNAs may play a role in ESC decidualization.

Expression pattern of *Hand2os1* in mice uteri during early pregnancy

To better evaluate the physiological function of differentially expressed lncRNA *Hand2os1* in RNA-seq data, RNAscope was performed to analyze the spatiotemporal distribution of *Hand2os1* in the uteri of pre-implantation pregnant mice (Fig. 4). Although *Hand2os1* could be detected using qRT-PCR in day 1 pregnant uteri, positive staining was too low to be detected by RNAscope (Figs 1 and 4). A weak *Hand2os1* signal was detected in a sub-population of ESCs near the luminal epithelium on day 2 of pregnancy. *Hand2os1* gradually increased and was primarily localized in

Figure 3 Relative levels of differentially expressed lncRNAs in mice uteri under artificial decidualization were detected by qRT-PCR. Rplp0 was used as a reference gene for normalization. *Prl8a2* was detected as a reliable decidualization marker. The data are expressed as the mean $\pm$ S.E.M. of three replicates, and the bar bearing different superscripts indicates a significant difference between the mean values ($P < 0.05$).

the sub-epithelial stromal cells from days 3 to 5 of pregnancy. A strong *Hand2os1* signal was observed in the antimesometrial deciduas, particularly those in the PDZ, which was well formed on day 6 of pregnancy. On days 7–8, *Hand2os1* was highly expressed in almost all areas of the endometrial stroma, including the antimesometrial and mesometrial deciduas. Notably, *Hand2os1* was mainly localized in the nuclei of stromal and decidual cells. No obvious *Hand2os1* signal appeared in the luminal epithelium or glandular epithelium from days 1 to 8 of pregnancy. The qRT-PCR results also confirmed that *Hand2os1* expression was highly elevated on day 4 of pregnancy and reached a peak on days 6 and 7, alongside the process of decidualization (Fig. 1). The spatiotemporal expression

pattern of *Hand2os1* during early pregnancy led to the hypothesis that *Hand2os1* may be a critical regulator of stromal cells during decidualization.

Expression of *Hand2os1* under artificial decidualization and pseudopregnancy

Consistent with the results of days 1 to 5 of pregnancy, *Hand2os1* signal was detected in the stromal cells of the non-injected uterine horn, whereas no positive signal was observed in the luminal and glandular epithelium cells (Fig. 5A). Meanwhile, high expression of *Hand2os1* was observed in the decidualized cells following artificial decidualization. The expression levels of *Hand2os1* in the deciduoma were also significantly

Figure 4 RNAscope of the *Hand2os1* expression pattern in mouse uteri on days 1 to 8 of pregnancy. Day 1 to day 8 (D1–D8) pregnant uterine sections were stained with a *Hand2os1* probe. Positive expression resulted in a brown color. L, luminal epithelium; S, stroma; G, glandular epithelium; M, myometrium; AM, antimesometrial region; MM, mesometrial region; C, conceptus; PDZ, primary decidual zone; SDZ, secondary decidual zone; D, decidual cells.

Figure 5 *Hand2os1* expression in mice uteri under artificial decidualization and pseudopregnancy. (A) *Hand2os1* expression and localization in the uterine horn under artificial decidualization detected by RNAscope; non-injected uterine horn served as a control. (B) RNAscope analysis of endometrial *Hand2os1* expression in a pseudopregnant mouse, day 1 (PD1), PD4, and PD6. G, glandular epithelium; L, luminal epithelium; S, stromal cells; D, decidual cells; M, myometrium.

higher than those in the control group, indicating that *Hand2os1* expression in decidua was not dependent on the presence of embryos ($P < 0.05$, Fig. 3). In addition, the pseudopregnancy model further confirmed that the expression of *Hand2os1* in these stromal cells

was not dependent on the embryo from days 1 to 6 of pseudopregnancy (Fig. 5B).

Regulation of *Hand2os1* expression by ovarian steroid hormones

To address the influence of E_2 and P_4 on uterine *Hand2os1* expression, we employed an ovariectomized mouse model injected with E_2 , P_4 , E_2 plus P_4 , or P_4 plus RU486. No *Hand2os1* signal was observed in the control or E_2 treatment mice (Fig. 6); however, expression of *Hand2os1* in ESCs was significantly stimulated by P_4 treatment, whereas RU486 markedly reduced the P_4 -dependent activation of *Hand2os1* stromal expression (Figs 6 and 7A, B). Similar to P_4 treatment only, *Hand2os1* expression was also significantly observed in ESCs by a combination of E_2 and P_4 , implying that E_2 does not have an antagonistic effect on the P_4 upregulation of *Hand2os1* expression (Figs 6 and 7A). On the basis of the previously mentioned observations, we subsequently detected the expression of *Hand2os1* at different time courses following P_4 treatment. The *Hand2os1* levels in ESCs significantly increased following P_4 injection for 24 h, with peak levels at 3 h (Figs 6 and 7C).

Figure 6 RNAscope of *Hand2os1* expression in the uterus after ovariectomized mice were treated with sesame oil (control), E_2 , P_4 , a combination of E_2 and P_4 , or P_4 plus RU486. A peptidylprolyl isomerase B (Ppib) probe was used to replace the *Hand2os1* probe as a positive control. L, luminal epithelium; G, glandular epithelium; S, stromal cells.

Figure 7 Hormonal regulation of *Hand2os1* expression detected by qRT-PCR. (A) The expression levels of *Hand2os1* in the uterus of ovariectomized mice after treatment with sesame oil (con), E_2 , P_4 , or a combination of E_2 and P_4 for 24 h. (B) *Hand2os1* expression in ovariectomized mouse uteri after injection of sesame oil (con), P_4 , RU486, or P_4 plus RU486. (C) *Hand2os1* expression in ovariectomized mouse uteri after injection of P_4 for 3, 6, 12, and 24 h. (D) *Hand2os1* expression in the uterine stromal cells treated with P_4 for 3, 6, 12, and 24 h or P_4 plus RU486 for 6 h. Values represent mean $\pm$ S.E.M. of three independent experiments. Each experiment was repeated at least three times. Student's *t*-test analysis was used to calculate the differences in (A) and (B). * $P < 0.05$ was considered statistically significant. One-way analysis of variance was used to calculate the differences in (C) and (D); bars with different letters are significantly different ($P < 0.05$).

To further support the previous mentioned results, we treated ESCs with P_4 *in vitro* and showed that *Hand2os1* expression was distinctly up-regulated during 24 h of P_4 treatment, with peak levels observed at 6 h; in contrast, RU486 markedly reduced the stimulatory effects of P_4 on *Hand2os1* expression ($P < 0.05$, Fig. 7D). The results suggest that the induction of *Hand2os1* expression by P_4 had been depended on the PR.

Effects of *Hand2os1* on decidualization *in vitro*

To further verify the role of *Hand2os1* in ESC decidualization, we silenced and overexpressed the *Hand2os1* gene in cultured primary ESCs ($P < 0.05$; Figs 8A and 9A). The results showed that, although no significant difference was observed in the heart and neural crest derivatives-expressed transcript 2 (*Hand2*) mRNA levels, silencing of *Hand2os1* expression significantly reduced the differentiation markers for *Prl8a2* and *Prl3c1* mRNA levels in ESCs during decidualization

in vitro ($P < 0.05$, Fig. 8B, C and D). Conversely, overexpression of *Hand2os1* was able to increase the mRNA expression of *Prl8a2* and *Prl3c1* genes ($P < 0.05$, Fig. 9B and C). Moreover, the expression levels of *Hand2* was not affected by *Hand2os1* overexpression (Fig. 9D). These results demonstrate that *Hand2os1* promoted the expression of decidualization-related genes in ESCs and influenced the process of decidualization *in vitro*.

Discussion

To date, genome-wide analysis of mouse lncRNAs in pre-implantation embryonic development has been widely reported (Xue *et al.* 2013, Yan *et al.* 2013, Liu *et al.* 2018). lncRNAs are emerging as potentially important regulators in early-stage embryonic developmental processes, including oocyte maturation, zygotic genome activation, and mitochondrial functions (Qiu *et al.* 2016). Furthermore, lncRNAs may potentially represent attractive biomarkers for developing non-invasive tests for the prediction of oocyte/embryo quality and pregnancy outcome (Bouckenheimer *et al.* 2016). Successful implantation depends on effecting a synchronized dialog between embryos and the maternal endometrium. Because of less conserved sequencing across phylogenetically related species in terms of nucleotides, the predicted novel lncRNAs cannot currently be inferred by sequence similarity searching (Necsulea *et al.* 2014). The landscape of lncRNA data sets in endometrial tissue during embryo implantation has, to date, remained nearly completely unexplored. In our previous study, we performed a systematic analysis of lncRNAs in pregnant mouse uteri on days 4 and 5 at implantation and inter-implantation sites using RNA-seq and identified a total of 7764 putative lncRNAs (Wang *et al.* 2017). During days 6 to 8 of pregnancy, the uterus undergoes a series of dramatic changes in response to an implanting embryo, including stromal proliferation and differentiation into decidual cells, which is a critical process accompanying embryo implantation. Therefore, the purpose of this study was to compile a complete list of lncRNAs expressed during decidualization. We performed deep transcriptome sequencing for mouse endometrium samples collected on days 4, 6, and 8 of pregnancy, respectively. Finally, a total of 2332 putative lncRNA transcripts were identified with high confidence at the whole-genome level, and 510 of these were identified as novel lncRNAs, of which 91% (464) were classified as lincRNAs.

In recent years, the roles of several lncRNAs in decidualization have been reported. The expression profiling of lncRNAs between the decidua of human preeclamptic and normal pregnancy were identified by RNA-seq (Tong *et al.* 2018). In human decidualization, lncRNA *HK2P1* plays a critical role through accelerating glycolysis via regulation of *HK2* expression, effected via competition for miR-6887-3p binding sites

Figure 8 siRNA-mediated knockdown of *Hand2os1* regulated the ESC decidualization-related genes' expression during decidualization *in vitro*. Mouse ESCs collected on day 4 of pregnancy were transfected with scrambled siRNA (si-NC) and *Hand2os1* siRNA (si-*Hand2os1*); then decidualization was induced with P_4 (1 μ M) and E_2 (10 nM) up to 72 h. qRT-PCR analysis was conducted for *Hand2os1* (A), *Prl8a2* (B), *Prl3c1* (C), and *Hand2* (D) expression, using RNA collected from the above-noted decidual ESCs at various times. The siRNA treatment reduced *Hand2os1* lncRNA levels by ~50%. GAPDH was used as a reference gene for normalization. The data are presented as means $\pm$ S.E.M. for three independent experiments; * P < 0.05, ** P < 0.01 was compared with the si-NC group.

(Lv *et al.* 2018). The ncRNA *LINC00473* expression is highly induced *in vitro* in human decidualization, via treatment with db-cAMP, and knockdown of *LINC00473* attenuates the expression of several crucial decidual transcription factors, as well as *WNT4* (Liang *et al.* 2016). Unfortunately, non-homologous sequencing of lncRNA *HK2P1* and *LINC00473* in human tissue was found in our RNA-seq results. *Dio3os* was strongly expressed in the decidual stromal cells and may be important for mouse decidualization via regulation of *Dio3* expression (Deng *et al.* 2014). Consistent with report, *Dio3os* was shown to be significantly up-regulated in mouse endometrium from pregnancy days 6 to 8, according to both the RNA-seq and qRT-PCR results in our study. Meanwhile, *Dio3os* was also highly expressed in artificial and *in vitro* decidualization. A microarray experiment reported that *Gdap10* was highly expressed in acetaminophen-induced liver injury (Kim *et al.* 2017). Hepatocyte chromosome polyploidization is an important response to liver injury. The polyploidy in decidual cells appear

to be crucial for successful embryo implantation and the support of embryo growth during early pregnancy. In our study, *Gdap10* was significantly up-regulated alongside the process of decidualization *in vivo* and *in vitro*, suggesting that *Gdap10* may be involved in differentiation and polyploid formation in mouse ESCs.

With the exception of higher expression in adult mouse heart tissue, northern blot analysis showed *Hand2os1* transcript to also be more strongly expressed in the uterus and placenta tissue (Anderson *et al.* 2016). In addition, *Hand2os1* was identified in human term placenta by RNA-seq analysis and displayed higher expression levels in female placenta compared with those in male placenta (Anderson *et al.* 2016). In this study, RNAscope and qRT-PCR further revealed *Hand2os1* to be specifically expressed in the ESCs of mice uteri and was significantly up-regulated during decidualization in normal pregnant and artificial decidualization models. Similarly, elevated expression levels of *Hand2os1* were also observed in decidualizing stromal cells, with a

Figure 9 The effects of *Hand2os1* overexpression on the expression of *Hand2os1* (A), *Prl8a2* (B), *Prl3c1* (C), and *Hand2* (D) detected by qRT-PCR. Mouse ESCs collected on day 4 of pregnancy were transfected with the control vector (pc-DNA3.1) and recombinant vector (pc-*Hand2os1*) and then decidualization was induced with P_4 (1 μ M) and E_2 (10 nM) for 24 h. The results were normalized to GAPDH. The data are presented as the mean $\pm$ S.E.M. of three independent experiments, and the bar bearing different superscripts indicate significant differences (P < 0.05).

time-dependent increase, after ESCs were induced for *in vitro* decidualization. Likewise, inhibition of *Hand2os1* with specific siRNA was able to reduce the expression of decidualization markers *Prl8a2* and *Prl3c1*. Conversely, overexpression of *Hand2os1* was able to augment the expression of *Prl8a2* and *Prl3c1*, which further verified the effects of *Hand2os1* during decidualization. It is known that *Hand2* is essential for embryo implantation and decidualization (Bhurke *et al.* 2016). Selective knockout of the *Hand2* gene in mouse uterine tissue led to implantation failure (Li *et al.* 2011). A primary study found that *Hand2os1* and *Hand2* were co-regulated by a bidirectional promoter with asymmetrical activity (Voth *et al.* 2009, Anderson *et al.* 2016). Recently, studies indicated that promoter DNA hypermethylation may be involved in co-down-regulation of *Hand2os1* and *Hand2* in endometrial adenocarcinoma (Yang *et al.* 2018). However, *Hand2* was not regulated by *Hand2os1* in transcription level (Voth *et al.* 2009, Yang *et al.* 2018). The results of *Hand2os1* KO and *Hand2os1*^{+/-} *Hand2*^{+/-} mice revealed that the expression of *Hand2* in embryonic heart development depended on the transcription of *Hand2os1* in cis but that the mature *Hand2os1* transcript was not required for this to occur (Anderson *et al.* 2016). In addition, knockdown or overexpression of mature *Hand2os1* transcripts in HL-1 cells did not alter *Hand2* mRNA expression (Anderson *et al.* 2016). Our results also showed that silencing or overexpression of *Hand2os1* did not change mRNA expression levels of *Hand2* in ESCs during decidualization *in vitro*. However, whether *Hand2os1* also regulates decidualization at the transcriptional level through *Hand2* requires further study. Interestingly, similar to the *Hand2* gene, increasing expression of *Hand2os1* in the mouse uterus during decidualization was not dependent on the presence of a conceptus (Huyen & Bany 2011).

In the current study, on days 6 and 8 of pregnancy, when the level of maternal P₄ was high, *Hand2os1* was strongly expressed in mice uteri, implying a possible correlation between *Hand2os1* expression and P₄ levels during decidualization. In fact, P₄ significantly induced *Hand2os1* expression in the ESCs in ovariectomized mice. Additionally, PR antagonist RU486 was able to abrogate the induction of P₄ on *Hand2os1* expression during decidualization, both *in vivo* and *in vitro*. In addition, our results showed that E₂ had no significant effect on *Hand2os1* expression and that *Hand2os1* expression was not changed by a combination of E₂ and P₄, relative to P₄ treatment only, indicating that E₂ appeared to have no antagonistic effect on P₄ up-regulation of *Hand2os1* expression.

In conclusion, the first comprehensive annotation of lncRNAs in pregnant mouse uteri during decidualization was presented in this paper using deep RNA-seq analysis. Accordingly, this paper provides a valuable resource of functional lncRNAs associated with endometrial decidualization. Additionally, P₄-induced *Hand2os1*

was specifically expressed in ESCs in a PR-dependent manner and dramatically increased as they underwent decidualization. Although *Hand2os1* is involved in the regulation of mouse decidualization, its precise molecular mechanism still requires additional research.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0401>.

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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Author contribution statement

P L and Y J designed the experiments and bioinformatics analysis; R C and Y J analyzed the data and drafted the manuscript; Y J, T Y, and R Z collected the samples and performed the experiments; S L, X G, and C S prepared figures and performed part of the data analysis. A W reviewed the manuscript. All authors have read and approved the final manuscript.

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