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Expression of Hedgehog Family Genes in the Rat Uterus During Early Pregnancy

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Abstract. Hedgehog (Hh) plays a pivotal role in various tissues during embryonic development, tissue homeostasis and tumorigenesis. In mammals, Hh exists in three homologs: Desert hedgehog (*Dhh*), Indian hedgehog (*Ihh*) and Sonic hedgehog (*Shh*). In this study, we cloned full-length cDNAs encoding *Dhh* and *Ihh* from the rat uterus. Their amino acid sequences have a high homology with those of the mouse and human. In addition, the changes of Hh gene expression in the rat uterus during early pregnancy were analyzed. The results showed that all three hedgehog mRNAs were detected in the rat uterus at the proestrus stage and during early pregnancy (1.5, 3.5, 5.5 and 7.5 days post coitus: dpc). *Ihh* mRNA expression varied and peaked at 3.5 dpc in the luminal and glandular epithelium. Expression was decreased on 5.5 dpc with the exception of sustained expression in the glandular epithelium. Despite such *Ihh* variability, the expressions of *Dhh* and *Shh* mRNA remained unchanged. This indicated that *Ihh* was mainly expressed in the rat uterus during early pregnancy. Moreover, the Hh target gene (glioma-associated oncogene homolog 1; *Gli1*) was also highly expressed at 3.5 dpc in the epithelium and periepithelial stroma in a manner similar to the temporal pattern of *Ihh* expression. This suggests that *Ihh* signaling axis play a role in the rat uterus during early pregnancy. In summary, our results elucidate that *Ihh* is a predominant Hh protein in the rat uterus during early pregnancy and that other Hhs have the potential to be expressed. This observation will help to elucidate the basic molecular mechanism of rat uterus during early pregnancy.

Key words: Hedgehog, Implantation, Rat, Uterus

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Pregnancy in mammals is accompanied by dramatic remodeling of uterine tissue, which provides optimal conditions for subsequent embryo implantation and development. In early pregnancy, the mammal's endometrial epithelial and stromal cells undergo rapid and coordinated proliferation and differentiation in response to steroid hormones, 17 β -estradiol (E₂) and progesterone (P₄) [1, 2]. In rodents, E₂ stimulates epithelial cells to initiate proliferation on days 1 to 3 of pregnancy. Then P₄, in combination with continuation of E₂ stimuli, triggers epithelial cell differentiation and stromal cell proliferation. By day 4 or 5, the epithelial and stromal cells are tidily differentiated in final preparation for embryo attachment and implantation [3]. As a result, the endometrium acquires uterine receptivity, allowing for various reactions such as implantation and decidualization. However, in spite of all we know regarding the many aspects of the receptive uterus, the frequency of occurrence of female infertility begs a deeper understanding, particularly, of the details of hormonal control of the molecular and cellular mechanisms involved. These things remain unclear, in part, due to the nature of the intricate signaling hierarchy process and complex dynamics of subsequent morphological change. In addition, these signal axes have not only many steps but also con-

tain aspects of epithelial-stromal reciprocal interaction. Therefore, a stereoscopic viewpoint of cell-to-cell cross-talk is needed to address the molecular pathways required to prepare for the ability of the uterus to provide for successful implantation.

A progesterone receptor (*Pgr*), which is both a cognate receptor of P₄ and a nuclear receptor, acts as a transcription factor to regulate target genes by binding to a specific ligand. This plays a pivotal role in remodeling of the uterus as a transducer of P₄ action. *Pgr* knockout in mice causes a complete failure of decidualization [4]. Takamoto *et al.* [5] compared *Pgr* knockout mice with wild-type mice in an attempt to define not only the physiological phenotype but also the molecular pathways involved. They found that a gene known as Indian hedgehog (*Ihh*) was stimulated by P₄ in the uterus. On day 3 of pseudopregnancy when the P₄ level becomes high, *Ihh* mRNA expression rose in both the luminal and glandular epithelium. In addition, both the hedgehog (Hh) receptor (patched 1; *Ptc1*) and a signal transducer of Hh (glioma-associated oncogene homolog 1; *Gli1*) were expressed in the epithelium and periepithelial stroma [6, 7]. These observations suggested that P₄-responsive *Ihh* mediates intracellular communication between epithelial-stromal compartments in the pregnant uterus. Previously it was difficult to analyze the function of *Ihh* because ablation of *Ihh* was lethal to the embryo [8]. More recently, however, a conditional mutation of *Ihh*, which can eliminate *Ihh* just after the *Pgr* promoter switches on, has been used to inhibit embryo implantation

Table 1. Sequences of the forward primers, reverse primers and probes used for real-time PCR

Gene	Forward (5'–3')	Reverse (5'–3')	Probe (5'–3')
<i>Dhh</i>	cgtgtcgggtcaaagctgat	agttccctcagcccttc	ggaggctg (#75*)
<i>Ihh</i>	cccacgttcattgctctgt	aggaaagcagccactgtc	gttggtg (#83)
<i>Shh</i>	ggaccgcagcaagtatgg	gccacggagttctctgct	tggctgtg (#76)
<i>Gli1</i>	ctggagggtctgcgtgtag	catggtgtctcagcgaagg	ccagggcc (#81)
<i>Hprt</i>	gaccggttctgcatctgcg	acctggttcacataactac	agtcccg (#95)

* Universal Probe Library probe number (Roche).

and decidualization [9]. In terms of its physiologic characteristics, this case is similar to that observed in earlier *Pgr* knockout mice [4]. This also confirmed *Ihh* as a major mediator of *Pgr*. Moreover, many downstream pathways of *Ihh* have been tested, but no notable factors have yet been discovered, and at present there remains little known regarding its downstream components.

Ihh is a member of the Hh family. Hh is a crucial morphogen of organogenesis and tumorigenesis [10]. It was first identified in *Drosophila* as a single gene and as a factor of segmentation. In mammals, there are three Hh homologs: the aforementioned Indian Hedgehog (*Ihh*), Desert hedgehog (*Dhh*) and Sonic hedgehog (*Shh*). Hh signaling is transmitted by two receptors, the twelve-transmembrane receptor Ptc and the seven-transmembrane receptor smoothened (Smo). Under the conditions of Hh binding to Ptc, Smo is released from the inhibitory action of Ptc. The signal-transducer of Hh protein, transcription factor Gli, is then translocated into the nucleus to regulate target gene transcription. This series of the Hh signaling pathway is wide-ranging through epithelial-mesenchymal communication resulting in cell proliferation, differentiation, apoptosis and migration. Unless Hh is expressed precisely, target cells become cancerous. Generally, Hh plays a role in various tissues during embryonic development. *Ihh* acts on osteogenesis and yolk sac formation [11, 12]. *Dhh* maintains germ cell survival together with normal testicular and prostate development [13, 14]. Finally *Shh* regulates left-right asymmetry, the polarity of limbs, foregut development, neuronal development and lung development [15–19]. Interestingly, Hh genes are expressed in a coordinated manner in some areas of reproductive organs including the prostate and ovaries [20]. However, there is not an obvious expression profile of the Hh family genes in the uterus with the exception of *Ihh*.

The Hh family genes are the key regulators of the endometrium to achieve receptivity for implantation by mediating intracellular communication between epithelial-stromal compartments. The present study was performed to further investigate the expressions of the three Hh family genes in the rat uterus. We first confirmed the sequences of rat *Ihh* and *Dhh* cDNA because they have previously only been analyzed *in silico*. We then investigated the expressions of all three Hh mRNAs and the Hh target gene *Gli1* by quantitative real-time PCR and *in situ* hybridization in the rat uterus during early pregnancy.

Materials and Methods

Animals

This research was conducted using Wistar strain rats raised in

our own laboratory (Department of Animal and Marine Biore-source Sciences, Graduate School of Agriculture, Kyushu University). The rats were housed under temperature- and light-controlled conditions (lights on from 0800 to 2000 h) with free access to food and water. The stages of the estrus cycles in each rat were determined by the vaginal smear method. Adult female rats were mated with males, and the day on which spermatozoa were found in the vaginal smear was designated 0.5 days post coitus (dpc). All experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals (Graduate School of Agriculture, Kyushu University) and with the approval of the Kyushu University Laboratory Animal Care and Use Committee.

Sequence determination and motif search

Rat *Ihh* and *Dhh* cDNA were amplified by specific primer sets from reverse transcribed uterine cDNA using an oligo (dT) primer or an inner sequences primer. Specific primer sets were designed by predicted sequences (*Ihh*, GenBank nucleotide sequence database genbank accession No. XM_001060415; *Dhh*, XM_001062733). After electrophoresis and extraction from agarose gel by MagExtractor –PCR & Gel Clean up– (Toyobo, Tokyo, Japan) for each PCR product, amplicons were then subcloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA). Sequencing was handled using a GenomeLab DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's protocol, followed by sequencing with a CEQ 8800 system (Beckman Coulter). An online signal peptide search, the signalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>), Motif search software, a NCBI Conserved Domain search (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and ExPASy PROSITE (<http://www.expasy.ch/prosite/>) were used for analysis of deduced amino acid sequences.

RNA extraction, reverse transcription (RT) and quantitative real-time PCR

Total RNA was extracted from homogenized uteri at the proestrus stage (P), 1.5, 3.5, 5.5 and 7.5 dpc using a Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) with RQ1 RNase-Free DNase (Promega) treatment, according to the manufacturer's protocol. RNA quality was assessed by ethidium bromide agarose gel electrophoresis and spectrophotometric UV absorbance at 260/280 nm. One microgram of each RNA sample was reverse transcribed with a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) using an oligo-dT primer in a 20 μ l solution. Real-time PCR was carried out using a Roche Light Cycler 1.5 system (Roche). A Taqman Master kit (Roche) in combination with

Fig. 1. Rat Hh family proteins. Rat IHH and DHH sequences were identified, and rat SHH sequence was cited from genbank (NM_017221). Amino acid residues are numbered on the right. Conserved amino acid residues are indicated by asterisks below the alignment. Hh proteins consist of a signal peptide, ligand peptide and Hint module. Rat hedgehog proteins are well conserved, especially in the ligand region.

Each experiment was repeated three times. The results are expressed as a ratio against the proestrus stage and are expressed as means $\pm$ SEM. Values were analyzed by ANOVA followed by the Student-Newman-Keuls test using StatView (Abacus Concepts, Berkeley, CA, USA). Differences were considered significant at the level of $P < 0.05$.

Fig. 2. Expressions of Hh genes in the rat uterus. A) Expression analysis of three Hh mRNAs as detected by RT-PCR. Total RNA was extracted from the rat uterus at 3.5 dpc. β -actin was used as an internal control. PCR was conducted using first-strand cDNA samples with (+) and without (-) RT. Lane M, DNA molecular weight standard; lane C, β -actin. B) *In situ* hybridization analysis of Hh mRNA expressions in the rat uterus at 3.5 dpc. Sections were hybridized with each probe. The hybridization signals were then detected using an anti-digoxigenin alkaline phosphatase conjugate (a and e, antisense *Dhh*; b and f, antisense *Ihh*; c, antisense *Shh*; d, sense *Ihh*). GE, glandular epithelium; LE, luminal epithelium; S, stroma; M, myometrium. The scale bars represent 100 μ m.

Results

Rat *IHH* and *DHH*

Rat *Ihh* and *Dhh* cDNA were amplified by RT-PCR using specific primers designed from predicted sequences. The deduced amino acid sequences of IHH, DHH and SHH had also a high degree of similarity, particularly in the Hh signaling ligand peptide region (Fig. 1). The Hh peptides consisted of an N-terminus signal peptide, ligand peptide and Hint module. Furthermore, the deduced amino acid sequences were highly homologous to the orthologs of mouse and human. Rat IHH shared 97% and 94% homology with mouse and human IHH, respectively. Likewise, rat DHH shared 100 and 97% homology with mouse and human DHH, respectively. Hh homologs were conserved well except for slight c-terminus diverseness.

Temporal expression of Hh genes in the rat uterus

To examine which Hh mRNAs are expressed in the rat uterus, we analyzed the expression of rat Hh mRNAs by RT-PCR (Fig. 2A). As expected, a strong signal of *Ihh* mRNA was detected in the uterus on 3.5 dpc. In addition, a weak signal of *Dhh* mRNA was detected, and *Shh* mRNA was barely detectable. Furthermore, only *Ihh* mRNA was detected in the luminal and glandular epithelium by *in situ* hybridization, and *Dhh* and *Shh* were not detected in the endometrium (Fig. 2Ba–d). Hh mRNAs were not detected by *in*

Fig. 3. Expression profiles of Hh genes in the rat uterus during early pregnancy. A) Changes in the expressions of Hh mRNAs analyzed by quantitative real-time PCR. Total RNA was extracted from the rat uterus at the proestrus stage (P) and at 1.5, 3.5, 5.5 and 7.5 dpc. *Hprt* was used as an internal control. The results are expressed as a ratio against the proestrus stage and are expressed as means $\pm$ SE (n=3). Values with different superscripts are significantly different (P<0.05). B) *In situ* hybridization analysis of *Ihh* mRNA expression in the rat endometrium during early pregnancy (a, 1.5 dpc; b, 3.5 dpc; c, 5.5 dpc). Sections were hybridized with *Ihh* probe. The hybridization signals were then detected using an anti-digoxigenin alkaline phosphatase conjugate. GE, glandular epithelium; LE, luminal epithelium; S, stroma. The scale bars represent 100 μ m.

situ hybridization in the myometrium of the rat uterus (Fig. 2Be and f). The changes in Hh mRNA expression during early pregnancy were analyzed by real-time PCR (Fig. 3A). In this study, all three mRNAs were detected in the rat uterus. *Ihh* mRNA expression in the 3.5 dpc uterus was particularly increased with about 5-fold higher expression than that in the 1.5 dpc uterus. The expression of *Ihh* mRNA decreased gradually to the same basal level as the proestrus stage at 7.5 dpc. On the other hand, the expressions of *Dhh* and *Shh* mRNAs did not change from the non-pregnant stage, proestrus cycle, through to 7.5 dpc. *In situ* hybridization analysis also showed a transient increase of *Ihh* mRNA expression during early pregnancy (Fig. 3B). No *Ihh* mRNA signal was detected in the epithelium or stroma at 1.5 dpc. However, a strong signal of *Ihh* mRNA was apparent in both the luminal epithelium and glandular epithelium on 3.5 dpc. On 5.5 dpc, expression was decreased in the luminal epithelium, but expression remained in the glandular epithelium.

Expression of *Gli1* gene during early pregnancy

Although *Ihh* mRNA was expressed in the luminal epithelium, expression downstream of Hh remained unclear. Therefore, we analyzed the expression of the Hh target gene *Gli1* by real-time PCR (Fig. 4A). *Gli1* mRNA was highly expressed on 3.5 dpc (at a level approximately 6-fold higher than that of the proestrus stage) and then decreased completely on 7.5 dpc in a manner similar to the

Fig. 4. Expression profile of the *Gli1* gene in the rat uterus during early pregnancy. **A)** Change in the expression of *Gli1* mRNA analyzed by quantitative real-time PCR. Total RNA was extracted from the rat uterus at the proestrus stage (P) and at 1.5, 3.5, 5.5 and 7.5 dpc. *Hprt* was used as an internal control. The results are expressed as a ratio against the proestrus stage and are expressed as means $\pm$ SE ($n=3$). Values with different superscripts are significantly different ($P<0.05$). **B)** *In situ* hybridization analysis of *Gli1* mRNA expression in the rat endometrium during early pregnancy (a, 1.5 dpc; b and d, 3.5 dpc; c, 5.5 dpc). The sections were hybridized with a sense (d) or antisense (a, b, c) *Gli1* probe. The hybridization signals were then detected using an anti-digoxigenin alkaline phosphatase conjugate. GE, glandular epithelium; LE, luminal epithelium; S, stroma. The scale bars represent 100 μ m.

temporal pattern of *Ihh* expression. Furthermore, expression of the *Gli1* mRNA was observed in the epithelium and periepipithelial stroma of the endometrium (Fig. 4B). However, *Gli1* mRNA was only weakly detected in the stroma distant from the epithelium.

Discussion

Hh essentially functions in various processes during embryonic development and tumorigenesis. Dysregulation of Hh signaling

leads to a number of critical human disorders. *Shh* mutation has been reported in the case of holoprosencephaly and cardiovascular disease [21, 22]. Hh also controls not only embryonic development but also homeostasis in the adult gut [23]. In addition, overexpression of Hh signaling results in cancer [24]. During the gestation period, it has been reported that Hh signaling plays an important role in the mouse endometrium through epithelial-stroma cross-talk, resulting in tissue remodeling of the uterus. In the present study, we demonstrated that Hh mRNAs are expressed in the rat uterus. In particular, transient expression of *Ihh* mRNA was observed in the 3.5 dpc uterus. These results are the first findings concerning Hh gene expressions in the uterus of pregnant rats and reflect those of previous reports in the mouse and hamster [5, 6, 25]; they also imply that Hh may play a significant role in mammals during the implantation phase.

In mammals, the Hh family includes *Ihh*, *Dhh* and *Shh*. Despite the importance of Hh signaling, rat *Ihh* and *Dhh* has only been analyzed *in silico* [26]. In postgenomic analysis, computer analysis is useful to determine orthologs and expression in tissues by EST. *In silico* analysis is highly capable of detecting exon-intron boundaries. Moreover, it can be used to evaluate both the transcription and translation starting positions and their stop positions. However, the entire sequences of rat *Ihh* and *Dhh* cDNA had not yet been identified. Therefore, the sequences were precisely confirmed in this study. Our results and previous reports show that Hh proteins consist of a signal peptide, ligand peptide and Hint module. In general, the Hh peptide is translated as an immature precursor [27]. The Hh precursor enters the secretion pathway with cleavage of a signal peptide. The autoprocessing reaction is subsequently achieved through catalyzation and mediation of a Hint module to cholesterol, followed by palmitoylation of the N-terminus. Finally, the fully-processed Hh ligand peptide is secreted from the producing cell. Rat *IHH*, *DHH* and *SHH* are all highly similar and well conserved, although their tissue distributions differ. Small differences include *SHH* being slightly longer in terms of its Hint module and the existence of an HPLGMXXXXXS motif in its C-terminus; these differences, together with those of *IHH*, might contribute to the diverseness of tissue distribution. Such diversity, however, is clearly not attributed to these differences alone. Analysis of the 5'-UTR promoter and 3'-UTR regions is important for understanding the mechanism of tissue distribution.

Interestingly, Hh has a significant effect in reproductive organs. For example, Hh signaling regulates not only organogenesis but also germ cell survival in the testis [13]. In addition, three Hh genes are co-expressed in the ovaries, and both *Ihh* and *Dhh* gene expression levels change dynamically [20]. Hh gene expression in the pregnant rat uterus has not been reported with the exception of *Ihh*. In the present study, the expression patterns of three Hh ligands were analyzed by real-time PCR. Not only *Ihh* mRNA expression, but also *Dhh* and *Shh* mRNA expressions, were detected in the uterus. However, the behavior of the expressions remained constant and did not fluctuate with the exception of *Ihh*. Furthermore, *in situ* hybridization analysis did not detect any signals with the *Dhh* and *Shh* probes. These results suggest that *Ihh* is mainly expressed in the rat uterus during early pregnancy and that the expression levels of *Dhh* and *Shh* mRNAs might be at a low

level and not induced by factors of pregnancy, including P₄ and E₂. Although their expressions might be low in the uterus at the proestrus stage or during early pregnancy, the potential roles of *Dhh* and *Shh* remain unclear.

In mice, *Ihh* mediates tissue remodeling in the pregnant uterus by the action of P₄ [5–7, 9]. In this study, quantitative analysis of Hh mRNA expression was conducted in the rat uterus. Similar to the reports in mice, *Ihh* mRNA was expressed in the luminal and glandular epithelium on 3.5 dpc and in the glandular epithelium on 5.5 dpc. The stage expressing *Ihh* mRNA corresponded with the dpc which, in turn, was correlated with increases in the P₄ level. Our quantitative results show that *Ihh* mRNA expression was high on 3.5 dpc, indicating that *Ihh* gene expression is only induced by P₄-signaling and is not induced by copulation stimuli or embryonic attachment. In spite of the high P₄ level maintained from 3.5 to 7.5 dpc, expression of *Ihh* mRNA was transient and was decreased at 7.5 dpc. This may be due to the action of E₂ on 3.5 dpc. Ovariectomized mice treated with both P₄ and E₂ show no expression of *Ihh* gene [6]. Wakitani *et al.* [28] reported that leukemia inhibitory factor (*Lif*) can induce *Ihh* expression during the implantation period. As *Lif* is induced by E₂, the tissue remodeling of the uterus mediated by *Ihh* must include the involvement of not only P₄ but also E₂. More investigation is needed to elucidate this concept.

In the present study, the expression of *Gli1* mRNA, one of the target genes of Hh, was analyzed in the rat uterus. The Gli family consists of three homologs in mammals, and it has been reported that the *Gli1* is transiently and synchronously expressed together with *Ihh* during early pregnancy in the mouse endometrium [6]. Our results in the rat uterus support the reports in the mouse. *Gli1* mRNA was expressed in the epithelium and periepithelial stroma. Little is known about the target genes of the transcription factor *Gli1* after translocation into the nucleus. To elucidate the function of *Ihh* during early pregnancy, further studies are required to investigate the relation between *Gli1* and endometrial decidualization.

In conclusion, Hh genes were expressed in the rat uterus during the uterine remodeling period. *Ihh* acts as a mediator of the P₄-dependent action of the endometrium in both mice and hamsters and may play a crucial role in beginning reconstruction of the uterus to prepare for embryo implantation, not only in rodents but also in other mammalian species [5, 6, 9, 25]. Understanding and controlling the Hh signaling pathway in the uterus may improve the fecundity rate of animal production and in turn lead to benefits in human reproductive health.

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