***Gja1* acts downstream of *Acvr1* to regulate uterine decidualization via *Hand2* in mice**

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Abstract

Although *Gja1* has been proved to play an important role in uterine decidualization, its regulatory mechanism remains largely unknown. Here, we showed that *Gja1* was highly expressed in the decidual cells and promoted the proliferation of uterine stromal cells and expression of *Prl8a2* and *Prl3c1*, which were two well-known differentiation markers for decidualization. Further analysis revealed that *Gja1* might act downstream of *Acvr1* and cAMP to regulate the differentiation of uterine stromal cells. Administration of cAMP analog 8-Br-cAMP to *Acvr1* siRNA-transfected stromal cells resulted in an obvious increase of *Gja1* expression, whereas PKA inhibitor H89 impeded the induction of *Gja1* elicited by *Acvr1* overexpression, indicating that cAMP–PKA signal mediates the regulation of *Acvr1* on *Gja1* expression. In uterine stromal cells, knockdown of *Gja1* blocked the cAMP induction of *Hand2*. Moreover, siRNA-mediated downregulation of *Hand2* impaired the stimulatory effects of *Gja1* overexpression on the expression of *Prl8a2* and *Prl3c1*, whereas constitutive expression of *Hand2* reversed the inhibitory effects of *Gja1* siRNA on stromal differentiation. Meanwhile, *Gja1* might play a vital role in the crosstalk between *Acvr1* and *Hand2*. Collectively, *Gja1* may act downstream of cAMP–PKA signal to mediate the effects of *Acvr1* on the differentiation of uterine stromal cells through targeting *Hand2*.

Key Words

- *Gja1*
- *Acvr1*
- *Hand2*
- decidualization
- uterine stromal cell

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Introduction

Decidualization, a process where uterine stromal cells undergo extensive proliferation and differentiation into decidual cells, is essential for continued embryonic development and successful pregnancy (Dey *et al.* 2004, Zhang *et al.* 2013). Inadequate decidualization causes embryo miscarriage and early pregnancy loss irrespective of whether development of the blastocyst is normal (Kommagani *et al.* 2013). It has been previously reported that activin A receptor type 1 (*Acvr1*, also referred to as *Alk2*) played an important role in modulating uterine decidualization. Conditional ablation of *Acvr1* in the

uterus exhibited decidualization failure in response to an artificial stimulus (Clementi *et al.* 2013). Although microarray analysis of oil-induced deciduoma in *Acvr1*-deficient mice has demonstrated that decidualization-related gene heart and neural crest derivatives expressed transcript 2 (*Hand2*) was downregulated (Huyen & Bany 2011, Clementi *et al.* 2013), the underlying molecular mechanism of *Acvr1* remains poorly understood.

Gap junctions, which are membranous channels for the exchange of small molecules directly between/among adjacent cells or between cells and their extracellular

environment, are critical for decidualization, because its blockade could suppress the proliferation and differentiation of uterine stromal cells (Yu *et al.* 2011, 2014a, Diao *et al.* 2013, Winterhager & Kidder 2015, Zappitelli *et al.* 2015). Gap junction protein alpha 1 (*Gja1*), also known as connexin 43 (*Cx43*), was the principal and most well-studied component of the gap junctions. Loss of *Gja1* expression in mouse uterus led to severe fertility defects due to the impaired stromal responsiveness to implanting embryo and decidualogenic stimulus accompanied with defective angiogenesis (Laws *et al.* 2008). Similar results have been obtained in human endometrial stromal cells in which attenuation of *Gja1* expression disrupted the gap junctional communication between neighboring cells and impeded the differentiation of stromal cells (Laws *et al.* 2008, Yu *et al.* 2011). However, there is still very limited information available regarding the regulatory mechanism of *Gja1* in the process of decidualization.

The present study was undertaken to examine the expression of *Gja1* in mouse uterus during the pre-implantation period, explored its effects on the proliferation and differentiation of uterine stromal cells and focused on analyzing the interplay of *Gja1*, *Acvr1*, cAMP and *Hand2* in stromal differentiation. The results showed that *Gja1* was highly expressed in the decidual cells and induced the proliferation and differentiation of uterine stromal cells. Furthermore, *Gja1* might act downstream of cAMP–PKA signal to mediate the effects of *Acvr1* on the differentiation of uterine stromal cells by targeting *Hand2*.

Materials and methods

Animal

Matured Kunming white strain mice (6–8 weeks old) were caged in a controlled environment with a cycle of 14L:10D. All animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University. To confirm reproducibility of results, at least three mice per group were used in each stage or treatment in this study.

Pregnancy and pseudopregnancy

Adult female mice were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy by cocaging, respectively (day 1 = day

of vaginal plug). On days 1–4, pregnancy was confirmed by recovering embryos from the oviducts or uterus. The implantation sites on day 5 were identified by intravenous injection of 0.1 mL of 1% Chicago blue (Sigma) in 0.85% sodium chloride.

Delayed implantation and activation

To induce delayed implantation, pregnant mice were ovariectomized under ether anesthesia at 08:30–09:00 h on day 4 of pregnancy. Progesterone (1 mg/mouse; Sigma) was injected subcutaneously to maintain delayed implantation from days 5 to 7. Estradiol-17 β (25 ng/mouse, Sigma) was given to progesterone-primed delayed implantation mice to activate blastocyst implantation. The mice were killed to collect uteri 24 h after estrogen treatment. The implantation sites were identified by intravenous injection of Chicago blue solution. Delayed implantation was confirmed by flushing the blastocysts from the uterus.

Artificial induced decidualization

Artificial decidualization was induced by intraluminally infusing 25 μ L of sesame oil into one uterine horn on day 4 of pseudopregnancy, whereas the contralateral uninjected horn served as a control. The mice were killed to collect uteri at 24, 48, 72 or 96 h after artificial-induced decidualization. Decidualization was confirmed by weighing the uterine horn and histological examination of uterine sections.

In situ hybridization

Total RNAs from the mouse uteri were reverse-transcribed and amplified with *Gja1* primers. *Gja1* forward primer 5'-GACTGCGGATCTCCAAAATA and reverse primer 5'-CTGTAATTCGCCAGTTTGT were designed according to *Mus musculus* gap junction protein, alpha 1 (*Gja1*) gene (GenBank accession number NM_010288). The amplified fragment (167 bp) of *Gja1* was cloned into pGEM-T plasmid (pGEM-T Vector System 1; Promega) and verified by sequencing. *Gja1*-containing plasmid was amplified with the primers for T7 and SP6 to prepare templates for labeling. Digoxigenin (DIG)-labeled antisense and sense cRNA probes were transcribed *in vitro* using a DIG RNA labeling kit (Roche Diagnostics GmbH).

Frozen sections (10 μ m) were mounted on 3-aminopropyltriethoxy silane (Sigma)-coated slides

and fixed in 4% paraformaldehyde solution in PBS. Hybridization was performed as described previously (Tian *et al.* 2013). Sections were counterstained with 1% methyl green. The positive signal was visualized as a dark brown color. The sense probe was also hybridized and served as a negative control. There was no detectable signal from sense probes.

Real-time PCR

Total RNAs from mouse uteri or cultured cells were isolated using TRIPURE reagent (Roche) according to the manufacturer's instructions, digested with RQ1 deoxyribonuclease I (Promega) to remove genomic DNA and reverse-transcribed into cDNA with M-MLV reverse transcriptase (Promega). Reverse transcription was performed at 42°C for 60 min with 2 µg total RNA in 25 µL volume. For real-time PCR, cDNA was amplified using FS Universal SYBR Green Real Master (Roche) on BIO-RAD

CFX96™ Real-Time Detection System. The conditions used for real-time PCR were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Water was used to replace template cDNA for negative control. All reactions were run in triplicate. The result was analyzed using CFX Manager Software. After analysis using the $2^{-\Delta\Delta C_t}$ method, data were normalized to *Gapdh* expression. Primer sequences for real-time PCR were listed in Table 1. Specificities of all primers were confirmed based on the agarose gel electrophoresis, sequencing and melting curves analysis.

Isolation of uterine stromal cells

Uterine stromal cells from day 4 of pregnancy were isolated by enzymatic digestion as previously described (Tian *et al.* 2013) and cultured with DMEM nutrient mixture F-12 Ham (Sigma) with 10% heat-inactivated fetal bovine serum (Life Technologies).

Table 1 Primers for real-time PCR.

Gene	Primer sequence	Accession number	Size (bp)
<i>Gja1</i>	CGGAAGCACCATCTCCAAC CCACGATAGCTAAGGGCTGG	NM_010288	110
<i>Ccna1</i>	GCCCCAGCTGGATGAGTTT AGGAGGAATTGGTTGGTGGTT	NM_007628	132
<i>Ccnb1</i>	CTGAGCCTGAGCTGAACCT AGCCCCATCATCTGCGTCT	NM_172301	191
<i>Ccnb2</i>	GCTAGCTCCCAAGGATCGTC CTGCAGAGCTGAGGGTTCTC	NM_007630	141
<i>Ccnd1</i>	GGGATGTGAGGGAAGAGGTGA GCAGCGAAAACAACGTGAAA	NM_007631	154
<i>Ccnd3</i>	CCTCCTACTTCCAGTGCGTG GGCAGACGGTACCTAGAAGC	NM_007632	195
<i>Ccne1</i>	AATGGATGGTTCCGTTCCG TGGGTCTGGATGTTGTGGG	NM_007633	103
<i>Cdk1</i>	CTGGGCACTCTAACAACGAAG TCCAAGCCGTTCTCGTCCAG	NM_007659	128
<i>Cdk2</i>	ACAGGGCAAGGTGAAAGAC AGGAGGACGGCAATGAGG	NM_016756	183
<i>Cdk4</i>	GTGGCTGAAATTGGTGTCGG TAACAAGGCCACCTCACGAA	NM_009870	156
<i>Cdk6</i>	TCCTGCTCCAGTCCAGCTAT CCACGTCTGAACCTCCACGA	NM_009873	108
<i>Prl8a2</i>	AGCCAGAAATCACTGCCACT TGATCCATGCACCCATAAAA	NM_010088	119
<i>Prl3c1</i>	GCCACACGATATGACCGGAA GGTTTGGCACATCTTGGTGTT	NM_001163218	162
<i>Acvr1</i>	TTGCGCGGAGAAGGACTC GCTGCATAGCAGATTGGGC	NM_001110205	178
<i>Hand2</i>	CACAGAACAAGGCCAAAGGC CTTGTCGTTGCTGCTCACTG	NM_010402	161
<i>Gapdh</i>	GCCTTCCGTGTTCTACCC TGCTGCTTACCACCTTC	NM_008084	102

RNA interference

The small-interfering RNA (siRNA) duplexes for targeting *Gja1*, *Acvr1* and *Hand2* as well as a scrambled sequence (control siRNA duplex, negative control) were synthesized by GenePharma. The sequences were as follows: 5'-CUGGGUCCUUCAGAUCAUATT and 5'-UAUGAUCUGAAGGACCCAGTT (*Gja1* siRNA 1); 5'-CUCUCGCUCUGAAUAUCAUTT and 5'-AUGAUUAUCAGAGCGAGAGTT (*Gja1* siRNA 2); 5'-CCUGCCGCAAUUAACAACAATT and 5'-UUGUUGUAAUUGCGGCAGGTT (*Gja1* siRNA 3); 5'-GGGUCGCUUUCAGGUUUAUTT and 5'-AUAACCUGAAAGCAGCCCTT (*Acvr1* siRNA 1); 5'-GGAAGUUUAAGAGACGCAATT and 5'-UUGCGUCUCUUAACUUCCTT (*Acvr1* siRNA 2); 5'-CUGUGAUGUUGAGGCAUGATT and 5'-UCAUGCCUCAACAUCACAGTT (*Acvr1* siRNA 3); 5'-GCUACAUCGCCUACCUCUATT and 5'-AUGAGGUAGGCGAUGUAGCTT (*Hand2* siRNA 1); 5'-CCUUCAGGCGGAGAUCAATT and 5'-UUGAUCUCCGCCUUGAAGGTT (*Hand2* siRNA 2); 5'-GGAAGAAAGAGCUGAAUGATT and 5'-UCAUUCAGCUCUUUCUUCCTT (*Hand2* siRNA 3); 5'-UUCUCCGAACGUGUCACGUTT and 5'-ACGUGACACGUUCGGAGAATT (nonspecific scrambled siRNA, negative control). Transfections for siRNA were performed according to Lipofectamine 2000 protocol. After transfection with *Gja1* siRNA, *Acvr1* siRNA

or *Hand2* siRNA, uterine stromal cells were collected at 24 h.

Plasmid construction and transfection

Full-length *Gja1*, *Acvr1* and *Hand2* cDNA fragments were amplified by PCR from mouse uterus using the following primers: *Gja1* (5'-AAGCTT (Hind III) ATGGGTGACTGGAGCGCCTTG and 5'-CTCGAG (Xho I) TTAATCTCCAGGTCATCAGG), *Acvr1* (5'-AAGCTT (Hind III) ATGGTCGATGGAGTAATGATC and 5'-GATATC (EcoR V) ACAAGGTCAACAGTCAGTT) and *Hand2* (5'-GAATTC (EcoR I) ATGAGTCTGGTGGGGGGCTT and 5'-AAGCTT (Hind III) TCCTCTTCTTCACTGCTTG). The amplified products were purified and cloned into pGEM-T vector. The pGEM-T-*Gja1*, pGEM-T-*Acvr1*, pGEM-T-*Hand2* and pcDNA3.1 vector were cut by Hind III/Xho I, Hind III/EcoR V or EcoR I/Hind III (TaKaRa) at 37°C for 1 h, and then, the fragments were ligated into pcDNA3.1 with T4 ligase (Promega) at 4°C overnight to construct pcDNA3.1-*Gja1*, pcDNA3.1-*Acvr1* and pcDNA3.1-*Hand2*, respectively. An empty pcDNA3.1 expression vector was served as control. Transfection of uterine stromal cells was performed according to the manufacturer's protocol for Lipofectamine 2000 (Invitrogen). After transfection with control plasmid (empty pcDNA3.1 vector) or overexpression plasmid, stromal cells were collected at 24 h.

Cell proliferation

Proliferation assays were performed using MTS reagent (Promega) according to the manufacturer's directions. Uterine stromal cells were seeded at a density of 1×10^5 /well in 96-well plates and cultured in the DMEM/F12 medium containing 2% heat-inactivated FBS. After transfection with *Gja1* siRNA or overexpression plasmid, stromal cells were cultured for 24 h. Finally, 20 µL of MTS reagent was added to each well and incubated for 4 h. Absorbance was measured at 490 nm using a 96-well plate reader. Every experiment was performed in triplicate.

Intracellular cAMP measurement

The intracellular cAMP levels were measured using cAMP-Glo Assay (Promega) according to the manufacturer's protocol. After transfection with *Acvr1* siRNA or overexpression plasmid, uterine stromal cells were cultured for 24 h. After the removal of media, cells were

incubated with the induction buffer containing 100 µM Ro 20-1724 and 500 µM IBMX in the absence or presence of forskolin (2 µM) for 20 min, and then treated with cAMP-Glo Lysis Buffer accompanied by shaking. Finally, 80 µL Kinase-Glo Reagent was added to each well and incubated for 10 min after treatment of cAMP-Glo Detection Solution. Luminescence was measured with a plate-reading luminometer. Every experiment was performed in triplicate.

Statistics

All the experiments were independently repeated at least three times. Significance of difference between two groups was compared by Independent Samples *T* Test. The multiple comparisons were tested with one-way ANOVA. The differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS19.0 software program (SPSS).

Results

Gja1 mRNA expression during early pregnancy

In situ hybridization was used to examine the distribution of *Gja1* mRNA in mouse uterus. There was no obvious *Gja1* mRNA signal in the uteri from days 1 to 4 of pregnancy (Fig. 1B, C and D). On day 5 of pregnancy, *Gja1* mRNA was highly expressed in the subluminal stroma surrounding the implanting blastocyst, but weakly detected at the inter-implantation sites (Fig. 1E and F). From days 6 to 8 of pregnancy, a high level of *Gja1* mRNA signal was observed in the decidua (Fig. 1G, H and I). However, when *Gja1* antisense probe was replaced by *Gja1* sense probe, there was no corresponding signal in day 7 uterus (Fig. 1A). We next quantified the expression level of *Gja1* mRNA in mouse uterus by real-time PCR. The result showed that *Gja1* mRNA expression was gradually increased from days 1 to 8 of pregnancy and reached a peak on day 8 (Fig. 2A). Compared with the inter-implantation sites, *Gja1* mRNA expression was higher at implantation sites on days 5–8 of pregnancy (Fig. 2B).

Gja1 mRNA expression during delayed implantation and activation

We used a delayed implantation model to determine whether *Gja1* expression was dependent on the presence of an active blastocyst. The results found that there was no

Figure 1

In situ hybridization of *Gja1* expression in mouse uteri during early pregnancy on days 1 (B), 3 (C), 4 (D), 5 (E and F), 6 (G), 7 (H) and 8 (I). No hybridization signals were seen in mouse uterus on day 7 of pregnancy when DIG-labeled *Gja1* sense probe was used to replace the antisense probe as a negative control (A). 5-I, implantation site on day 5 of pregnancy; 5-N inter-implantation site on day 5 of pregnancy. Asterisks indicate embryo. Bar = 60 μm.

Figure 2

Real-time PCR analysis of *Gja1* expression in mouse uteri. (A) *Gja1* expression on days 1–8 of pregnancy. (B) *Gja1* expression at the implantation sites and inter-implantation sites on days 5–8 of pregnancy. (C) *Gja1* expression during delayed implantation and activation. (D) *Gja1* expression under artificial decidualization. Delay, delayed implantation; Activation, activation of delayed implantation by estrogen; Control, uninjected uterine horn; Oil, oil-induced decidualization. Data are shown mean $\pm$ S.E.M. Asterisks denote significance ($P < 0.05$).

Figure 3
In situ hybridization of *Gja1* expression in mouse uteri. (A) and (B) *Gja1* expression during delayed implantation and activation. (C, D, E, F, G and H) *Gja1* expression under artificial decidualization.

evident *Gja1* mRNA signal in the delayed uterus (Fig. 3A). After the dormant blastocyst was activated and implanted, *Gja1* mRNA was visualized in the subluminal stromal cells surrounding the implanting embryo (Fig. 3B). By real-time PCR analysis, a significantly high level of *Gja1* expression was detected in activated implantation uterus compared with delayed uterus (Fig. 2C).

***Gja1* mRNA expression under artificial decidualization**

Because *Gja1* was highly expressed in the decidua, we subsequently examined its expression in an artificial induced decidualization model. As expected, *Gja1* mRNA signal was clearly seen in the decidualizing stromal cells

after sesame oil was injected into uterine lumen, whereas scarcely found in the uninjected control uteri (Fig. 3C, D, E, F, G and H). Simultaneously, real-time PCR results also confirmed the higher expression of *Gja1* in the deciduoma compared with control, which gradually enhanced from 24 to 96 h as the decidua was developing and reached a maximum at 96 h after sesame oil injection (Fig. 2D).

Effects of *Gja1* on stromal cell proliferation and differentiation during decidualization

Based on the expression pattern of *Gja1* in mouse uterus, we assume that *Gja1* is important for mouse decidualization. As is known to us all, stromal cell proliferation is the first step of decidualization. To investigate the role of *Gja1*, we synthesized three different *Gja1* siRNA, transfected the uterine stromal cells and found that *Gja1* siRNA 1 and 2 could apparently downregulate the expression of *Gja1* mRNA compared with scrambled siRNA, but the former was more significant, whereas *Gja1* siRNA 3 did not change the expression of *Gja1* (Fig. 4A). Therefore, *Gja1* siRNA 1 was chosen for further experiments. After transfection with *Gja1* siRNA, proliferation of uterine stromal cells displayed a marked reduction compared with control (Fig. 4B). On the contrary, overexpression of *Gja1* could strengthen the expression of *Gja1* and proliferation activity of stromal cells (Fig. 4C and D). To further understand the molecular basis for the proliferative role of *Gja1*, we analyzed the effects of *Gja1* on the expression of cyclin A1 (*Ccna1*), *Ccnb1*, *Ccnb2*, *Ccnd1*, *Ccnd3*, *Ccne1*, cyclin-dependent kinase 1 (*Cdk1*), *Cdk2*, *Cdk4* and *Cdk6*. The results evidenced that silencing of *Gja1* could reduce the expression of *Ccnd3* and *Cdk4* in the uterine stromal cells, whereas overexpression of *Gja1* exhibited the opposite effects (Fig. 4E and F). *Gja1* did not impact the expression of *Ccna1*, *Ccnb1*, *Ccnb2*, *Ccnd1*, *Ccne1*, *Cdk1*, *Cdk2* and *Cdk6* in the uterine stromal cells (Fig. 4E and F).

To study the effects of *Gja1* on the differentiation of uterine stromal cells, we analyzed its effects on the expression of prolactin family 8, subfamily a, member 2 (*Prl8a2*) and prolactin family 3, subfamily c, member 1 (*Prl3c1*), which were two well-known differentiation markers for decidualization (Clementi *et al.* 2013, Li *et al.* 2016). After *Gja1* siRNA were introduced into the stromal cells, the expression of *Prl8a2* and *Prl3c1* was remarkably decreased (Fig. 4G). In contrast, constitutive expression of *Gja1* could remarkably enhance the expression of *Prl8a2* and *Prl3c1* mRNA in the stromal cells (Fig. 4H).

Figure 4

Effects of *Gja1* on the proliferation and differentiation of uterine stromal cells. (A) Effects of *Gja1* siRNAs on *Gja1* mRNA expression in the uterine stromal cells. After transfection with control siRNA, *Gja1* siRNA 1, siRNA 2 or siRNA 3, *Gja1* mRNA expression was determined by real-time PCR. (B) Effects of *Gja1* siRNA on the proliferation of uterine stromal cells. (C) *Gja1* expression after uterine stromal cells were transfected with *Gja1* overexpression plasmid. (D) Effects of *Gja1* overexpression on the proliferation of uterine stromal cells. (E) Effects of *Gja1* siRNA on the expression of *Ccna1*, *Ccnb1*, *Ccnb2*, *Ccnd1*, *Ccnd3*, *Ccne1*, *Cdk1*, *Cdk2*, *Cdk4* and *Cdk6*. (F) Effects of *Gja1* overexpression on the expression of *Ccna1*, *Ccnb1*, *Ccnb2*, *Ccnd1*, *Ccnd3*, *Ccne1*, *Cdk1*, *Cdk2*, *Cdk4* and *Cdk6*. (G) Effects of *Gja1* siRNA on the expression of *Prl8a2* and *Prl3c1*. (H) Effects of *Gja1* overexpression on the expression of *Prl8a2* and *Prl3c1*. NC, control siRNA; siGja1, *Gja1* siRNA; Con, empty pcDNA3.1 vector; Gja1, *Gja1* overexpression plasmid.

***Gja1* mediates the effects of *Acvr1* on the differentiation of stromal cells**

It is well known that *Acvr1* was required for uterine decidualization (Clementi *et al.* 2013). In the stromal cells, knockdown of *Acvr1* with specific siRNA could efficiently repress the expression of *Acvr1*, *Prl8a2*, *Prl3c1* and *Gja1*, whereas overexpression of *Acvr1* could augment their expression (Fig. 5A, Supplementary Fig. 1A, B, C and D, see section on supplementary data given at the end of this article). Neither attenuation nor overexpression of *Gja1* had obvious impact on the expression of *Acvr1* in the uterine stromal cells (Fig. 5B). We next tested whether *Gja1* might mediate the effects of *Acvr1* on the differentiation of stromal cells. After co-transfection with *Acvr1* overexpression plasmid and *Gja1* siRNA, the expression of *Prl8a2* and *Prl3c1* in the uterine stromal cells was evidently suppressed (Fig. 5C and D). Conversely, overexpression of *Gja1* could reverse the effects of *Acvr1* siRNA on the expression of *Prl8a2* and *Prl3c1* (Fig. 5E and F).

***Gja1* mediates the effects of cAMP on the differentiation of uterine stromal cells**

It has been well established that uterine decidualization was accompanied by elevated intracellular cAMP levels (Gellersen & Brosens 2003). In the uterine stromal cells,

cAMP analog 8-bromoadenosine cAMP (8-Br-cAMP, 500 μ M) stimulated the expression of *Gja1* in a time-dependent manner (Fig. 6A). As a ubiquitous second messenger, cAMP prominently activates protein kinase A (PKA) signal transduction pathway (Gellersen & Brosens 2003). Addition of PKA inhibitor H89 (10 μ M) could block the stimulation of 8-Br-cAMP on *Gja1* expression (Fig. 6B). To ascertain whether *Gja1* could mediate the effects of cAMP on the differentiation of uterine stromal cells, we transfected the stromal cells with *Gja1* siRNA, and then add 8-Br-cAMP and analyzed the expression of *Prl8a2* and *Prl3c1*. The results showed that siRNA-mediated attenuation of *Gja1* could impede the enhancement in the expression of *Prl8a2* and *Prl3c1* elicited by 8-Br-cAMP (Fig. 6C and D).

***Acvr1* regulates the expression of *Gja1* through cAMP–PKA signal**

After uterine stromal cells were treated with 8-Br-cAMP, *Acvr1* expression did not change with or without PKA inhibitor H89 (Fig. 7A and B). Knockdown of *Acvr1* with specific siRNA could reduce the cAMP level in the stromal cells in the absence or presence of forskolin, an agent known to increase intracellular levels of cAMP, whereas overexpression of *Acvr1* enhanced the intracellular cAMP level (Fig. 7C and D). We next study

Figure 5

Gja1 mediates the effects of *Acvr1* on the differentiation of uterine stromal cells. (A) Effects of *Acvr1* siRNA or overexpression on the expression of *Gja1*. (B) Effects of *Gja1* siRNA or overexpression on the expression of *Acvr1*. (C) and (D) *Gja1* siRNA blocked the effects of *Acvr1* overexpression on the expression of *Prl8a2* and *Prl3c1*. After co-transfection with *Acvr1* overexpression plasmid and *Gja1* siRNA, the expression of *Prl8a2* and *Prl3c1* was determined by real-time PCR. (E) and (F) overexpression of *Gja1* improved the effects of *Acvr1* siRNA on the expression of *Prl8a2* and *Prl3c1*. siAcvr1, *Acvr1* siRNA; Acvr1, *Acvr1* overexpression plasmid.

whether cAMP–PKA signal is involved in regulating the effects of *Acvr1* on the differentiation of uterine stromal cells. The results demonstrated that 8-Br-cAMP could improve the effects of *Acvr1* siRNA on the expression of *Prl8a2* and *Prl3c1* in the stromal cells, whereas PKA inhibitor H89 prevented the induction of *Prl8a2* and *Prl3c1* by *Acvr1* overexpression (Fig. 7E and F). Because *Acvr1* and cAMP–PKA signal modulated the expression of *Gja1* in the stromal cells, we surmised that regulation of *Acvr1* on *Gja1* expression was mediated by cAMP–PKA signal. Indeed, after transfection with *Acvr1* siRNA and then addition of 8-Br-cAMP, *Gja1* expression in the stromal cells was drastically elevated (Fig. 7E). In the meantime, administration of H89 to uterine stromal cells transfected with *Acvr1* overexpression plasmid resulted in a robust decline in the expression of *Gja1* (Fig. 7F).

Figure 6

Gja1 mediates the effects of cAMP on the differentiation of uterine stromal cells. (A) *Gja1* expression in the uterine stromal cells treated with 8-Br-cAMP for 6, 12 and 24 h. (B) *Gja1* expression after uterine stromal cells were treated with 8-Br-cAMP or both 8-Br-cAMP and H89. (C) and (D) *Gja1* mediated the effects of cAMP on the expression of *Prl8a2* and *Prl3c1*. After transfection with *Gja1* siRNA and addition of 8-Br-cAMP, the expression of *Prl8a2* and *Prl3c1* was determined by real-time PCR.

Gja1 regulates the differentiation of uterine stromal cells via *Hand2*

Because *Hand2* is expressed in the decidua and overlapped with *Gja1* (Huyen & Bany 2011), we assumed that there was a relationship between *Gja1* and *Hand2* during decidualization. To elucidate the relationship, we first examined the regulation of *Hand2* on *Gja1*. Treatment with siRNA targeted to *Hand2*, which efficiently suppressed the level of *Hand2* mRNA, led to a remarkable reduction in the expression of *Prl8a2* and *Prl3c1*, but had no obvious impact on the expression of *Gja1* (Fig. 8A, Supplementary Fig. 2A and B). Overexpression of *Hand2*, which could distinctly raise the expression of *Hand2*, *Prl8a2* and *Prl3c1* in the stromal cells, did not affect the expression of *Gja1* (Fig. 8A, Supplementary Fig. 2C and D). Afterward, the regulation of *Gja1* on *Hand2* was further determined. The result evidenced that *Hand2* expression was restrained by *Gja1* siRNA and augmented by *Gja1* overexpression (Fig. 8B). We next dissect whether *Hand2* may mediate the effects of *Gja1* on the differentiation of uterine stromal cells. Knockdown of *Hand2* in the stromal cells with specific siRNA could impair the effects of *Gja1* overexpression on the expression of *Prl8a2* and *Prl3c1*, whereas overexpression of *Hand2* compensated the effects of *Gja1* silencing on the expression of *Prl8a2* and *Prl3c1* (Fig. 8C, D, E and F).

Figure 7

Acvr1 regulates the expression of *Gja1* through cAMP-PKA signal. (A) *Acvr1* expression in the uterine stromal cells treated with 8-Br-cAMP for 6, 12 and 24 h. (B) *Acvr1* expression after uterine stromal cells were treated with 8-Br-cAMP, or both 8-Br-cAMP and H89. (C) Effects of *Acvr1* siRNA on the intracellular cAMP level in the absence or presence of forskolin. (D) Effects of *Acvr1* overexpression on the intracellular cAMP level in the absence or presence of forskolin. (E) cAMP reversed the effects of *Acvr1* siRNA on the expression of *Prl8a2*, *Prl3c1* and *Gja1*. (F) H89 impeded the effects of *Acvr1* overexpression on the expression of *Prl8a2*, *Prl3c1* and *Gja1*.

***Gja1* mediates the regulation of cAMP on *Hand2* expression**

As described previously, *Gja1* could mediate the effects of cAMP on the differentiation of stromal cells and adjust the expression of *Hand2*. Moreover, our previous study has tested that cAMP could facilitate the expression of *Hand2* in the uterine stromal cells (Li *et al.* 2015). The siRNA-mediated downregulation of *Hand2* could alleviate the induction of 8-Br-cAMP on the expression of *Prl8a2* and *Prl3c1* (Fig. 8G and H). We next asked whether cAMP regulation of *Hand2* expression was mediated by *Gja1*. After transfection with *Gja1* siRNA and addition of 8-Br-cAMP, a drastic reduction of *Hand2* mRNA expression was noted in the uterine stromal cells (Fig. 8I).

Gja1* acts downstream of cAMP-PKA signal to mediate the regulation of *Hand2* by *Acvr1

As demonstrated previously, the effects of *Acvr1* on the differentiation of stromal cells were mediated by *Gja1*, which modulated the expression of *Hand2*. Based on the observation, we hypothesized that *Hand2* might mediate the action of *Acvr1* in stromal differentiation. To confirm the hypothesis, we first tested the interaction between *Acvr1* and *Hand2*. The result found that silencing of *Acvr1* with specific siRNA could downregulate the expression of *Hand2* in the uterine stromal cells, whereas constitutive expression of *Acvr1* had opposite effects (Fig. 9A). Neither inhibition nor overexpression of *Hand2* altered the expression of *Acvr1* in the stromal cells (Fig. 9B). Simultaneously, knockdown of *Hand2* could prevent the *Acvr1*-induced upregulation of *Prl8a2* and *Prl3c1* in the uterine stromal cells (Fig. 9C and D). Conversely, overexpression of *Hand2* could improve the expression of *Prl8a2* and *Prl3c1* in the *Acvr1* siRNA-transfected stromal cells (Fig. 9E and F). Together, these data imply that *Hand2* may be a downstream target of *Acvr1*. We next sought to determine whether the regulation of *Acvr1* on *Hand2* expression was mediated by *Gja1*. The result revealed that attenuation of *Gja1* expression in the stromal cells could abolish the induction of *Acvr1* on *Hand2* expression, whereas overexpression of *Gja1* rescued the inhibitory effects of *Acvr1* siRNA on *Hand2* expression (Fig. 10A and B). Additionally, we wonder whether cAMP-PKA signal is involved in regulating the effects of *Acvr1* on *Hand2* expression. Administered 8-Br-cAMP to stromal cells transfected with *Acvr1* siRNA resulted in a drastic increase in the expression of *Hand2* (Fig. 10C). In contrast, PKA inhibitor H89 eliminated the induction of *Hand2* by *Acvr1* overexpression (Fig. 10D).

Discussion

Gap junctions are essential for embryo implantation and decidualization, because its blockade led to an obvious reduction of the number of implanted embryos and the impairment of decidualization (Yu *et al.* 2011, 2014a, Diao *et al.* 2013, Winterhager & Kidder 2015). As a major gap junction protein, *Gja1* was weakly expressed in the mouse uteri on days 1–3 (prereceptive) and day 4 (receptive) of pregnancy and delayed implanting uteri, suggesting that *Gja1* is dispensable for the transformation of uterus into the receptive state during embryo implantation.

Figure 8

Gja1 regulates the differentiation of uterine stromal cells via *Hand2* and mediates the regulation of cAMP on *Hand2* expression. (A) Effects of *Hand2* siRNA or overexpression on the expression of *Gja1*. (B) Effects of *Gja1* siRNA or overexpression on the expression of *Hand2*. (C) and (D) *Hand2* siRNA weakened the effects of *Gja1* overexpression on the expression of *Prl8a2* and *Prl3c1*. (E) and (F) overexpression of *Hand2* improved the effects of *Gja1* siRNA on the expression of *Prl8a2* and *Prl3c1*. (G) and (H) *Hand2* mediated the effects of cAMP on the expression of *Prl8a2* and *Prl3c1*. (I) *Gja1* mediated the regulation of cAMP on *Hand2* expression. siHand2, *Hand2* siRNA; Hand2, *Hand2* overexpression plasmid.

Figure 9

Hand2 mediates the effects of *Acvr1* on the differentiation of uterine stromal cells. (A) Effects of *Acvr1* siRNA or overexpression on the expression of *Hand2*. (B) Effects of *Hand2* siRNA or overexpression on the expression of *Acvr1*. (C) and (D) *Hand2* siRNA weakened the effects of *Acvr1* overexpression on the expression of *Prl8a2* and *Prl3c1*. (E) and (F) overexpression of *Hand2* improved the effects of *Acvr1* siRNA on the expression of *Prl8a2* and *Prl3c1*.

The notion was also supported by previous reports, which conditional knockdown of *Gja1* in mouse uterus could normally initiate embryo implantation (Laws *et al.* 2008).

After the onset of embryo implantation, uterine stromal cells begin to undergo decidualization in response to the implanting embryo (Dey *et al.* 2004, Zhang *et al.* 2013). With the progression of decidualization, elevated expression of *Gja1* mRNA was observed in the decidual cells, suggesting a role of *Gja1* in decidualization. Stromal cell proliferation is the first step of decidualization (Chen *et al.* 2009, Zhang *et al.* 2013). Inhibition of *Gja1* by specific siRNA reduced the proliferation of uterine stromal cells, whereas overexpression of *Gja1* exhibited the opposite effects. Similarly, blockade of *Gja1*-mediated gap junction weakened the proliferation of human endometrial stromal cells (Yu *et al.* 2014a). It is well known that the proliferation of mammalian cells is controlled by cyclins and Cdks (Wang *et al.* 2010). In the uterine stromal cells, *Gja1* modulates the expression of *Ccnd3* and *Cdk4*, indicating that *Gja1* regulates stromal cells proliferation by targeting *Ccnd3* and *Cdk4*. Along with this noticeable proliferation, stromal cells differentiate into decidual cells (Zhang *et al.* 2013). Conditional deletion of *Gja1* impaired the differentiation of uterine stromal cells in mice (Laws *et al.* 2008). Similar results have been obtained in human endometrial stromal cells in which attenuation of *Gja1* expression could impede stromal differentiation (Laws *et al.* 2008,

Figure 10

Gja1 acts downstream of cAMP–PKA signal to mediate the regulation of *Acvr1* on *Hand2* expression. (A) *Gja1* siRNA abrogated the effects of *Acvr1* overexpression on *Hand2* expression. (B) Overexpression of *Gja1* rescued the effects of *Acvr1* siRNA on *Hand2* expression. (C) cAMP reversed the effects of *Acvr1* siRNA on *Hand2* expression. (D) H89 impeded the effects of *Acvr1* overexpression on *Hand2* expression.

Yu *et al.* 2011). The present study has reinforced the importance of *Gja1* in the differentiation of uterine stromal cells. Moreover, reduced *Gja1* expression was

Figure 11

Schematic diagram shows the involvement of *Gja1* during decidualization. *Gja1* may act downstream of cAMP–PKA signal to mediate effects of *Acvr1* on the differentiation of uterine stromal cells through targeting *Hand2*.

also noted in the decidua and endometrial stromal cells of women with recurrent early pregnancy loss and endometriosis, respectively (Nair *et al.* 2011, Yu *et al.* 2014b, Winterhager & Kidder 2015). Together these data demonstrate that *Gja1* plays an important role in the proliferation and differentiation of uterine stromal cells. Further analysis found that *Gja1* could regulate the expression of *Hand2* whose knockdown damaged the uterine stromal differentiation in both mice and human (Huyen & Bany 2011). Overexpression of *Hand2* could rescue the effects of *Gja1* siRNA on the expression of *Prl8a2* and *Prl3c1*, whereas attenuation of *Hand2* expression by siRNA prevented the upregulation of *Prl8a2* and *Prl3c1* elicited by *Gja1* overexpression. These results provide convincing evidence that *Hand2* is a critical mediator of *Gja1* function in the differentiation of uterine stromal cells.

It has previously been reported that *Gja1*, as the major component of gap junctions, might mediate the gap junctional communication among endometrial stromal cells (Laws *et al.* 2008, Yu *et al.* 2011). Intriguingly, the present study revealed that *Gja1* could modulate the expression of *Ccnd3*, *Cdk4* and *Hand2* genes. Simultaneously, there was a considerable amount of evidence to support the similar notion by these findings that deficiency of *Gja1* in the heart, testicular Sertoli cells, astrocytes and glioblastoma cells led to the downregulation and upregulation of numerous genes by microarray analysis (Huang *et al.* 2002, Iacobas *et al.* 2003, 2005, Giese *et al.* 2012). The reason was probably due to the interaction of *Gja1* with these proteins involved in gene transcription. Indeed, accumulating data have identified a lot of *Gja1*-interacting partners (Butkevich *et al.* 2004, Singh *et al.* 2005, Dai *et al.* 2007, Martins-Marques *et al.* 2015). However, the regulatory mechanisms of *Gja1* on the expression of *Ccnd3*, *Cdk4* and *Hand2* in uterine stromal cells remain to be determined.

It is generally accepted that *Acvr1* is of great importance to uterine decidualization (Clementi *et al.* 2013). In female mice with uterine-specific deficiency of *Acvr1*, stromal cells failed to undergo decidualization (Clementi *et al.* 2013). The present study found that the effects of *Acvr1* on the differentiation of uterine stromal cells were mediated by *Gja1*. Meanwhile, in the absence of *Acvr1*, *Hand2* expression in oil-induced deciduoma was significantly downregulated (Clementi *et al.* 2013). Consistent with the observation, *Hand2* was also regulated by *Acvr1* in the uterine stromal cells and has been proved be a crucial participant in *Acvr1*-mediated

stromal differentiation. Overexpression of *Gja1* could improve the expression of *Hand2* in the *Acvr1* siRNA-transfected stromal cells, whereas silencing of *Gja1* abolished the induction of *Acvr1* on *Hand2* expression, implying that *Gja1* may play an important role in the crosstalk between *Acvr1* and *Hand2*.

It has been well established that initiation of the decidual process requires elevated intracellular cAMP levels (Gellersen & Brosens 2003). In mouse embryonic stem cells, thymic epithelial cells and IMR-32 neuroblastoma cells, cAMP analog 8-Br-cAMP could upregulate the expression of *Gja1* (Arnold *et al.* 2005, Nihei *et al.* 2010, Yun *et al.* 2012). The similar result was also observed in the uterine stromal cells. As a ubiquitous second messenger, cAMP can prominently activate the PKA signal pathway (Gellersen & Brosens 2003). PKA inhibitor H89 abrogated the stimulation of 8-Br-cAMP on *Gja1* expression. Moreover, siRNA-mediated downregulation of *Gja1* expression greatly lessened cAMP-induced stromal differentiation as evidenced by the drastically reduced expression of *Prl8a2* and *Prl3c1*, suggesting *Gja1* requirement for the cAMP action on stromal differentiation. Further study found that 8-Br-cAMP could reverse the inhibitory effects of *Acvr1* siRNA on *Gja1* expression, whereas H89 eliminated the induction of *Gja1* elicited by *Acvr1* overexpression, indicating that cAMP–PKA signal is involved in the regulation of *Acvr1* on *Gja1* expression.

In summary, the present study unravels a linear pathway involving *Gja1*, *Acvr1*, cAMP and *Hand2* during decidualization. We show that *Gja1* may act downstream of cAMP–PKA signal to mediate the effects of *Acvr1* on the differentiation of uterine stromal cells through targeting *Hand2* (Fig. 11).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0583>.

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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