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The homologous genes Vangl1 and Vangl2 are required for embryo implantation in the uterus of mice during early pregnancy☆

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ABSTRACT

Vangl1 and Vangl2 are homologous genes belonging to the group of highly conserved planar cell polarity proteins. It has been shown that Vangl1 and Vangl2 are essential for embryonic development, cell adhesion, migration and polarity. We examined the expression of Vangl1 and Vangl2 in the uterus of mice during early pregnancy. They are upregulated in the endometrium of peri-implantation and reached the peak on D5. Vangl1 mRNA is widely distributed in the luminal epithelium, glandular epithelium and stromal cells in the endometrium, while its protein only appeared in the stromal cells. The localization of Vangl2 protein overlapped with its mRNA. In addition, expression of Vangl1 in the endometrium of pseudopregnant mice was lower than that of pregnant mice, whereas the level of Vangl2 was not significantly different, suggesting that expression of Vangl1 is induced by embryo. Further study showed that implantation would be suppressed after silencing expressions of Vangl1 and Vangl2 by uterine injection with antisense oligonucleotides. These findings suggest that Vangl1 and Vangl2 may play a key role in the embryo implantation of mice.

1. Introduction

The Wnt signaling pathway is an ancient and evolutionarily conserved pathway that regulates crucial aspects of cell fate determination, migration, polarity, neural patterning and organogenesis during embryonic development (Komiya and Habas, 2008). The extracellular Wnt signal stimulates several intracellular signal transduction cascades, including the canonical Wnt/β-catenin dependent pathway and the non-canonical β-catenin-independent pathway, which can be divided into the Planar Cell Polarity (PCP) pathway and the Wnt/Ca^{2+} pathway (Habas and Dawid, 2005). The aberrant expression of Wnt gene, as Wnt5a, Wnt11, and Wnt7a, would result in abnormal implantation and decidual dysfunction in mice (Mericskay et al., 2004). Wnt/β-catenin signaling is crucial for embryo implantation, and the dysregulation of β-catenin would affect glandular formation, decidualization and eventually lead to fertility (Jeong et al., 2009; Mohamed et al., 2005). The Wnt/PCP pathway, which can be stimulated by the non-canonical Wnt ligands, such as Wnt5a or Wnt11, is involved in tissue morphogenesis, directed cell migration, cell adhesion and polarity-cellular processes that are required for embryo implantation (Komiya and Habas, 2008; Wu et al., 2011). During embryo implantation, epithelial cells transit from a columnar to cuboidal phenotype approaching implantation, and junctions between neighboring epithelial cells are required to establish and maintain base-apical cell polarity (Etienne-Manneville, 2008; Nallasamy et al., 2012). However, it is unknown whether the Wnt/PCP signaling pathway directly participates in embryo implantation.

Mammalian Vangl proteins are highly conserved transmembrane (TM) membrane proteins, and they are composed of four putative TM domains in the N-terminus that were identified in two Vangl homologous genes in each species included Vangl1 and Vangl2 (Kibar et al., 2001). There is >80% consensus in the protein sequence of the cytoplasmic portion of Vangl1 and Vangl2, conferring structural homologies that reflect functional similarity (Torban et al., 2008). Vangl proteins play a key developmental role in the Wnt/PCP signaling pathway as they are constituent proteins in the Frizzled, Disheveled, Flamingo, Prickle, and Diego complexes (Adler, 2002; Mlodzik, 2002), regulating cytoskeleton, cell adhesion, migration, polarity and embryonic development (Wu et al., 2011). Vangl1 is expressed in the developing neural tube, and it is located in the floor plate and notochord (Torban et al., 2008). Vangl2 is expressed in the developing olfactory organs, the bronchial tree and some tube organs (Phillips et al., 2005; Henderson et al., 2006; Torban et al., 2007; Yates et al., 2010a,b). Loss-of-function in Vangl1 and Vangl2 would cause Neural Tube Defects (NTDs) during embryonic development. Recently, four mutation types of Vangl1 were identified in patients with NTDs, including familial types (V239I and

Abbreviations: NTDs, Neural Tube Defects; A-ODNS, antisense oligonucleotides; LE, luminal epithelium; GE, glandular epithelium; S, stromal; DE, decidua; EM, embryo.

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Table 1
Primers of Vangl1, Vangl2 and β-actin for real-time PCR.

Gene	GenBank accession no.	Sense primers (5′ → 3′)	Antisense primers(5′ → 3′)	Product size (bp)
Vangl1	NM_177545	AAGCAAAGAGCGGATGTG	CGATGGCAAGGTAGTGGA	196
Vangl2	NM_033509	TCAGGGTGACGGTTGACTT	CTTGTAGGAATAGCCCGAGTA	172
β-actin	NM_007393	CCTGAGGCTCTTTTCCAGCC	TAGAGGTCTTTACGGATGTCAACGT	120

R274Q), a sporadic type (M328T) and a spontaneous mutation (V239I) appearing in a familial setting (Reynolds et al., 2010). Furthermore, mutations in Vangl2 were heterozygous in fetuses with a cranial NTD: S84F (737C → T), R353C (1543C → T), and F437S (1796 T → C), with these mutations producing lethal effects on the embryo (Lei et al., 2010). The molecular mechanism for these defects is associated with Vangl1 and Vangl2 in the PCP pathway, directing embryonic convergent extension, polarized cell division, cell differentiation and cilia orientation (Barrow, 2006; Karner et al., 2006).

As a principal component of the Wnt/PCP signaling pathway, our aim is to study the role of Vangl1 and Vangl2 in embryo implantation. Because the mechanism of embryo implantation in mice is similar to human (Lee and DeMayo, 2004), mice were employed to carry out the study. We first detected the expression profile of Vangl1 and Vangl2 in the endometrium of pregnant and pseudopregnant mice. Further functional study was performed by uterine injection with antisense oligonucleotides (A-ODNS), and then counted the number of implantation sites to observe the effect of silencing their expression in endometrium on implantation.

2. Materials and methods

2.1. Animal models and experimental design

National Institutes of Health mice were obtained from the Animal Facility of Chong Qing Medical University, Chongqing, China (Certificate: SICXK[19] 2007 – 0001). 6–8 week old female mice (25–30 g) were caged in a specific pathogen-free animal room under a controlled environment, with a 14 h light and 10 h dark cycle. The estrus females were mated with fertile males or vasectomized males to induce pregnancy (D1 = day of positive vaginal plug) or pseudopregnancy (PD1 = day of positive vaginal plug). Pregnant mice were divided into six groups (D1, D2, D3, D4, D5 and D6), and pseudopregnant mice were divided into three groups (PD4, PD5 and PD6), (20 mice in each group). 20 pregnant mice on D3 were used for the Vangl1 and Vangl2 functional experiments. The mice in each group were killed in the morning for tissue collection. Trypan blue was injected into the tail veins to identify the implantation sites. Part of the mouse endometria tissue was collected and stored in liquid nitrogen for real-time quantitative polymerase chain reaction (qPCR) and Western blotting (WB), and the rest part of the uteri tissue was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry (IHC) and in situ hybridization (ISH). All animal procedures were approved by the Ethical Committee of Chongqing Medical University.

2.2. Real-time PCR

Total RNA was extracted from mouse endometrial tissues using Trizol reagent (Invitrogen, CA, USA), according to the manufacturer's instructions. Quantification and purity assessment were performed by optical density measurements at 260 and 280 nm. Integrity of the total RNA was examined by agarose gel electrophoresis. Total RNA was converted to cDNA and the expression of Vangl1 in the cDNA samples was determined using a SYBR Primescript™ RT-PCR kit (TAKARA, Dalian, China). β-Actin was used as a reference gene for each sample. Specific primers were designed (Shanghai GeneCore BioTechnologies Co., Ltd, China), and the primer sequences are shown in Table 1. The real-time PCR conditions were as follows: initial denaturation at 95 °C for 10 s, 40 cycles of 15 s at 95 °C (denaturation) and 30 s at 56 °C (annealing and extension). All real-time PCR experiments were repeated three times for each sample. Relative gene expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Expression levels of Vangl1 and Vangl2 were obtained by normalizing the amount of cDNA to that of β-actin.

2.3. In situ hybridization

In situ hybridization was performed using a universal digoxigenin-labeled probe hybridization kit (Beijing Dingguo Biosciences Inc., China). According to the manufacturer's instructions, sections (4 μm) were cut, deparaffinized and rehydrated. The endogenous peroxidase activity was quenched by incubation with 0.5% hydrogen peroxide in methanol for 30 min at room temperature, and then incubated with 50 μl proteinase K solution for 10 min at 37 °C. Sections were then washed three times for 3 min each and incubated with 50 μl of prehybridization solution for 3 h at 37 °C. After shaking off excess liquid, sections were incubated with hybridization solution containing 2 μg/ml of the hydridization probe (Invitrogen, CA, USA). The sequences for the digoxigenin-labeled probes for Vangl1 and Vangl2 were as follows: Vangl1: 5′-CCT GCG TTC GTG TTC GGC CTC TTCA TAA TAC AAC TCG TTG-3′; Vangl2: 5′-AGC AGG ATG AGC AGC TTG AAG GCC ACG GAG ATG AAG AGG C-3′ (Liu et al., 2008). Reaction conditions were as follows: initial denaturation at 95 °C for 5 s and overnight at 37 °C. The next day, the section was blocked for 30 min at 37 °C, followed by incubation for 1 h with rabbit anti-digoxin IgG and then incubated for 1 h with AP-Goat anti-rabbit IgG at 37 °C. The reaction product was detected using a BCIP/NBT Chromogenic agent kit (Boster, Wuhan, China). The localization of mRNA was analyzed and quantified using Medical Image Analysis Software (Beihang University, China), blue-violet staining was considered to be a positive signal.

2.4. Western blotting

Total proteins were extracted from the mouse endometrium using cell lysis buffer for Western blotting and immunoprecipitation (Beyotime, Jiangsu, China). The protein concentration was measured using the BCA Protein Assay kit (Beyotime, Jiangsu, China). Samples were boiled in 5× SDS sample loading buffer for 10 min and separated by SDS-polyacrylamide gel electrophoresis. The proteins were then transferred electrophoretically onto polyvinylidene difluoride membranes (BioRad, California, USA). The membrane was then blocked for 1 h at room temperature in PBST containing 5% skimmed milk powder. It was then incubated with anti-Vangl1 antibodies (1:500, sc-166844, Santa Cruz), anti-Vangl2 antibodies (1:500, sc-46561, Santa Cruz), or mouse monoclonal anti-β-actin antibodies (1:500,ZA-09,Zhongshan Golden Bridge Biotechnology Co., LTD., Beijing) overnight at 4 °C. Next, the membranes were washed for four times with PBST (5 min each time), followed by incubation for 1 h with a secondary antibody conjugated with horseradish peroxidase (Zhongshan Golden Bridge Biotechnology Co., LTD., Beijing). The membranes were washed three times in PBST and the antibodies were detected by enhanced chemiluminescence (ECL) using the appropriate ECL reagents (Merck Millipore, USA), and quantified by densitometry using Quantity One software (BioRad, California, USA).