Costimulatory Molecule CD28 Participates in the Process of Embryo Implantation in Mice

Reproductive Sciences
2014, Vol. 21(6) 686-695
© The Author(s) 2013
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1933719113512537
rs.sagepub.com

Shangjing Liu, MD¹, Junlin He, MD¹, Xuemei Chen, PhD¹,
Yubin Ding, PhD¹, Yanqing Geng, PhD¹, Mengyun Wu, MD¹,
Xueqing Liu, MD¹, and Yingxiong Wang, MD¹

Abstract

Embryo implantation is a complex process requiring reciprocal interactions between implantation-competent blastocysts and receptive uteri. Accumulating literatures have indicated that T cells are involved in this process. The first signal mediated by T-cell receptor/CD3 complex and the second signal delivered by costimulatory molecules are essential for the differentiation of T cell into an effector cell. Expression and function of CD28, an important costimulatory molecule, during early pregnancy in mice is still unclear. In the present study, we investigated the expression pattern of CD28 in mouse uterus during early pregnancy and pseudopregnancy by real-time quantitative polymerase chain reaction, Western blotting, in situ hybridization, and immunohistochemistry (IHC). We found that injection of the uterine horn with CD28 antisense oligodeoxynucleotides leads to a decreased number of implantation sites. The expression pattern of CD3 protein examined by IHC is similar to that of CD28. These findings suggest that CD28 participates in the process of embryo implantation in mice, which might play its role through delivering the second costimulatory signal.

Keywords

CD28, embryo implantation, costimulatory signal

Introduction

Embryo implantation is an essential and complex process in mammal reproduction, which involves preimplantation embryos developing to the blastocyst stage and then hatching from the zona pellucida to establish reciprocal interactions between the trophoblast and uterine luminal epithelium.¹ In mice, it is widely accepted that endometrium can receive embryo implantation during a limited period, called the implantation window.² The mouse uterus becomes receptive for blastocyst implantation on day 4 but becomes nonreceptive by day 5.³ Although the endocrine system plays a main role in preparing the endometrium for embryo implantation, there is evidence suggesting that the immune system also participates in this process.⁴ Accumulating literatures have confirmed that immune cells derived from pregnant mice directly promote endometrial differentiation during the peri-implantation period in a manner independent of the endocrine system.^{5,6} It is reported that lymphocytes had a specific expression in the endometrium at different phases of the menstrual cycle and might play an important role in embryo implantation.⁷ Together, these findings suggest that the maternal immune system is involved in embryo implantation.

A recent report has revealed that the composition and function of the immune cells are locally controlled by decidua that

surrounds the implanted embryos.⁸ Previous studies on lymphocytes in deciduas were mainly focused on natural killer (NK) cells, the most widely distributed immune cells in decidua.⁹ It is reported that decidual NK cells promoted immune tolerance and successful pregnancy.¹⁰ Nevertheless, there is little report on T cells in early pregnancy. It is now widely believed that naive CD4⁺ T cells require 2 distinct signals to proliferate and subsequently differentiate into the armed effector cells that mediate adaptive immunity.¹¹ In addition, previous studies have shown that expression of costimulatory molecules including CD28 arise in the in vitro model of mouse embryonic stem cells.¹² However, the expression of CD28 in mice uteri and its role in the process of embryo implantation are still unclear.

¹ Laboratory of Reproductive Biology, School of Public Health, Chongqing Medical University, Yuzhong District, Chongqing, People's Republic of China

Corresponding Author:

Xueqing Liu and Yingxiong Wang, Laboratory of Reproductive Biology, School of Public Health, Chongqing Medical University, No. 1 Yixueyuan Rd, Chongqing 400016, People's Republic of China.
Email: a68733172@online.cq.cn; wyx61221@aliyun.com

Table 1. The Sequence of Primers for qPCR.

Gene	Sequence of Primer: 5' to 3'	
CD28 (NM_007642.4)	Forward	TTTACCTATCAGCCCCAGTTTC
	Reverse	AGTTCCATTGCTCCTCTCGTT
β -Actin (NM_007393.3)	Forward	CCTGAGGCTCTTTTCCAGCC
	Reverse	TAGAGGTCTTTACGGATGTCAACGT

Abbreviation: qPCR, quantitative polymerase chain reaction.

Here, we examined the expression pattern of *CD28* in mouse uteri during early pregnancy and pseudopregnancy. We observed that *CD28* had a spatial temporal expression in mouse uterus and found that its expression at implantation sites (ISs) was much lower than that at interimplantation sites (IISs) on day 5. Our experiments also showed that injection of the uterine horn with *CD28* antisense oligodeoxynucleotides (A-ODNs) led to a decreased number of ISs. The expression pattern of *CD3* protein examined by immunohistochemistry (IHC) was similar to that of *CD28*. These findings suggest that *CD28* participates in the process of embryo implantation in mice, which might play its role through delivering the second costimulatory signal.

Materials and Methods

Animals and Tissue Collection

The 8-week-old female and male Kunming mice (25-30 g) were purchased from the Animal Facility of Chongqing Medical University (Certification No.: SCXK [YU] 20070001). The animal procedures were in accordance with the ethics committee of Chongqing Medical University (No. 20110016). The mice used in this study were maintained in no specific pathogen room in the experimental animal center of Chongqing Medical University. They were housed in plastic cages and exposed to a 12-hour light/12-hour dark cycle, with access to food and water ad libitum. The female mice were caged with fertile males or vasectomized males (2 weeks after vasectomy) to induce pregnancy or pseudopregnancy models,¹³ and the appearance of the vaginal plug was designated as day 1 (D1) of gestation. The pregnant mice were randomly assigned into 7 groups (D1-D7), and the pseudopregnant mice were assigned into 7 groups (Pd1-Pd7) containing 10 mice per group, with another 10 non-pregnant mice (D0 or Pd0) being used as controls. The other 60 mice were used to study the function of *CD28* by intrauterine injection experiment. After being anesthetized with 0.2 mL 10% chloral hydrate, the mice in each group were killed at 08:00 to 09:00 AM; 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 for IHC and in situ hybridization (ISH). The tissue of 10 mice in each group was divided into 3 tissue pools with uteri of 3 or 4 mice to meet the needs for qPCR and WB. The method of collecting ISs and IISs tissues was referred

to a previous report.¹⁴ The ISs on day 5 of pregnancy were identified by intravenous injection through tail vein of 0.1 mL 1% Chicago blue (Sigma, St Louis, Missouri).

Real-Time qPCR

Total RNA was extracted from the mouse endometrial tissues using RNAiso Plus Reagent (Takara, China) according to the manufacturer's protocol. Quantification and purity were assessed by optical density measurement at 260 and 280 nm. The integrity of the total RNA was examined by agarose gel electrophoresis. Reverse transcription was conducted in a 20- μ L reaction system using PrimeScript RT reagent Kit (Takara). The specific primers of the *CD28* and β -actin for qPCR were designed and synthesized by Sangon Biotech Co Ltd (Shanghai, China). The sequence of primers for qPCR is shown in Table 1. The PCR reaction was performed by Bio-Rad iQ 5 Real-Time PCR Detection System (Bio-Rad, Hercules, California). Experiments were performed in triplicate for each sample, and $2^{-\Delta\Delta C_t}$ method was used to calculate the relative expression of *CD28* in endometria on different pregnancy day with β -actin as an inner control.¹⁵

Western Blotting

Proteins were extracted from the endometria of mouse from each group using radioimmunoprecipitation assay lysis buffer containing 1% phenylmethanesulfonyl fluoride (Beyotime Biotechnology, China) to prevent the degradation of protein. Protein concentrations were determined by BCA method according to the manufacturer's instruction (Beyotime Biotechnology). Protein samples were electrophoresed through 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, Massachusetts). Immunoblotting was performed using a goat polyclonal anti-*CD28* antibody at a 1:200 dilution (sc-1624; Santa Cruz, Dallas, Texas). A mouse monoclonal β -actin antibody (clone: AC-15) at a 1:2000 dilution (A5441; Sigma, St Louis, Missouri) was used to equal loading of samples. A rabbit anti-goat immunoglobulin (Ig) G secondary antibody and a goat anti-mouse IgG secondary antibody were incubated to detect *CD28* and β -actin, respectively. The positive bands were detected by chemiluminescent reaction (Millipore). The image collection and densitometry analysis were performed by Quantity One version 4.6.2 analysis software (Bio-Rad).

In Situ Hybridization

Oligonucleotide probe of *CD28* labeled with digoxigenin was designed and synthesized by Dingguo BioTechnology Co Ltd (Beijing, China) and the sequence was 5'-AGAGC AGTGATGATGAGCAGGTAG-3'. Hybridization was performed using ISH Kit according to the manufacturer's protocol (Dingguo BioTech). Digoxigenin-labeled *CD28* oligonucleotide probe was used in this study. As a negative control (NTC), the probe was replaced with hybridization buffer. After hybridization, the signal was visualized with

Figure 1. Expression of *CD28* mRNA in the uteri during early pregnancy. A, Relative expression of *CD28* mRNA from D0 to D7 determined by qPCR. *, $P < .05$. B, Relative expression of *CD28* mRNA at implantation sites and interimplantation sites on D5 determined by qPCR. *, $P < .05$. C, The location of *CD28* mRNA determined using in situ hybridization (ISH) and blue stain means positive signal (200 $\times$). Bar = 100 μ m. IS indicates implantation sites; IIS, interimplantation sites; le, luminal epithelium; ge, glandular epithelium; s, stroma; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction.

5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Finally, sections were counterstained with kernechtrot and mounted with resinene. The images were captured by Olympus microscope (BX40; Olympus, Tokyo, Japan).

Immunohistochemistry

The tissue sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol, followed by antigen retrieval and cooling to room temperature. The following experiments were performed using Histostain-Plus Kits (ZSGB-BIO, China), and chromogenic reaction was performed with 3,30-diaminobenzidine (ZSGB-BIO) according to the manufacturer's protocol. The primary antibodies used in our study were a rabbit polyclonal anti-*CD28* antibody at a 1:80 dilution (bs1297R; Bioss, China) and a rabbit monoclonal anti-*CD3* antibody

(clone: SP7) at a 1:25 dilution (ab16669; Abcam, United Kingdom). As an NTC, primary antibodies were replaced with phosphate-buffered saline. The sections were counterstained with hematoxylin and mounted with resinene. The images were also collected by Olympus microscope (BX40; Olympus).

Injection

To determine whether *CD28* was involved in embryo implantation, 60 mice were given an intrauterine injection. The method was based on our previous research with some improvement.¹⁶ Specifically, 30 mice were injected with 10 μ g *CD28* reverse A-ODNs and 10 μ g *CD28* A-ODNs in the left and right side of the uteri individually at 19:00 to 20:00 PM on D3 of gestation. Another 30 mice were injected with 10 μ L ddH₂O and nothing in the left and right side of the uteri, respectively. The sequence

Figure 2. Expression of CD28 protein in the uteri during early pregnancy. A, The level of CD28 protein expression during D0 to D7 examined using Western blotting (WB). β-Actin was used as control. B, CD28 relative protein expression at IS and IIS on D5. C, A quantitative presentation of data shown in (A). *, $P < .05$. D, A quantitative presentation of data shown in (B). *, $P < .05$. E, The location of CD28 protein determined using immunohistochemistry (IHC) and yellowish-brown stain means positive signal (200 $\times$). Bar = 100 μ m. NTC indicates negative control; IS, implantation sites; IIS, inter-implantation sites; le, luminal epithelium; ge, glandular epithelium; s, stroma.

of *CD28* A-ODNs was 5'-AACAGCAGCCTGAGTGT CATTGTTC-3', and *CD28* reverse A-ODNs (as control) was 5'-CTTGTTACTGTGAGTCCGACGACAA-3', which were designed and synthesized by Shengbo Technology Co, Ltd (Chongqing, China). As the half-life of ODNs is between 24 and 48 hours,¹⁷ 20 mice (10 mice: left: reverse A-ODNs and right: A-ODNs; 10 mice: left: ddH₂O and right: nothing) were killed, respectively, at 24 hours (D4.5) and 48 hours (D5.5) after

injection to confirm whether the injection was successful. The other 20 mice were killed between 08:00 and 09:00 AM on D7 of pregnancy to record the number of ISs.

Statistics

The collected data were analyzed with the SPSS 13.0 (Chicago). The relative expression of messenger RNA

Figure 3. Expression of *CD28* mRNA in the uteri during early pseudopregnancy. **A**, Relative expression of *CD28* mRNA from Pd0 to Pd7 determined by quantitative polymerase chain reaction (qPCR). **B**, The location of *CD28* mRNA determined using in situ hybridization (ISH) and blue stain means positive signal (200 $\times$). Bar = 100 μ m. le indicates luminal epithelium; ge, glandular epithelium; s, stroma; mRNA, messenger RNA.

(mRNA) and protein between IS and IIS was determined by Student *t* test. The relative expression of mRNA and protein during early pregnancy and pseudopregnancy was determined by 1-way analysis of variance (ANOVA) test. The number of ISs after injection was determined by 1-way ANOVA test. Differences were considered statistically significant if $P < .05$. Data were expressed as the mean $\pm$ standard deviation.

Results

Expression of *CD28* mRNA in the Uteri During Early Pregnancy

To determine a possible role of *CD28* in uteri, we examined the expression level of *CD28* mRNA in endometria using real-time qPCR. Our results showed that during early pregnancy, the

CD28 mRNA level presented an upregulated trend around the implantation window and reached a peak on D5 of pregnancy and reduced thereafter (Figure 1A). The *CD28* mRNA level at IS was significantly lower than that at IIS on D5 of pregnancy ($P < .05$; Figure 1B). Figure 1C showed the *CD28* mRNA was detected in epithelium and stroma during early pregnancy. The expression of *CD28* mRNA increased gradually from D1 to D4 in the stroma of uterus and reached maximum on D5, thereafter decreased on D6 and D7.

Expression of *CD28* Protein in the Uteri During Early Pregnancy

Expression levels of *CD28* protein in endometria were examined by WB and IHC. During early pregnancy, the *CD28*

Figure 4. Expression of CD28 protein in the uteri during early pseudopregnancy. A, The levels of CD28 protein during Pd0-Pd7 examined using Western blotting (WB). β-Actin was used as control. B, A quantitative presentation of data shown in (A). *, $P < .05$. C, The locations of CD28 protein determined using immunohistochemistry (IHC) and yellowish-brown stain means positive signal (200×). Bar = 100 μm. NTC represents negative control. le, luminal epithelium; ge, glandular epithelium; s, stroma.

protein level presented an upregulated trend and reached a peak on D5 of pregnancy and reduced thereafter (Figure 2A). The CD28 protein level at IS was significantly lower than that at IIS on D5 ($P < .05$; Figure 2B). Figure 2C and D is the quantitative figure of Figure 2A and B, respectively. The locations of CD28 protein examined by IHC (Figure 2E) were correlated with the locations of *CD28* mRNA detected by ISH (Figure 1C).

Expression of CD28 mRNA in the Uteri During Early Pseudopregnancy

To further explore whether the expression of CD28 was induced by competent blastocysts, the expression pattern of *CD28* mRNA during early pseudopregnancy was tested using real-time qPCR. The levels of *CD28* mRNA on Pd4 were a little higher (no significant difference) than that of

Figure 5. Expression of CD3 protein in the uteri during early pregnancy. The location of CD3 protein determined using immunohistochemistry (IHC) and yellowish-brown stain means positive signal (200 $\times$). Bar = 100 μ m. NTC represents negative control. le, luminal epithelium; ge, glandular epithelium; s, stroma.

the other pseudopregnant days ($P > .05$; Figure 3A). Figure 3B shows that the *CD28* mRNA was detected in epithelium and stroma during early pseudopregnancy. The expression level of *CD28* mRNA was parallel to the results of real-time qPCR.

Expression of CD28 Protein in the Uteri During Early Pseudopregnancy

The expression level of CD28 protein during early pseudopregnancy was tested by WB. There was only a slight increase in CD28 protein on Pd4, and no significance was found among Pd1 to Pd7 of pseudopregnancy (Figure 4A and B). The location of CD28 protein examined by IHC was consistent with that of *CD28* mRNA (Figure 4C).

Expression of CD3 Protein in the Uteri During Early Pregnancy

Two distinct signals are essential for proliferation and differentiation of T cell into an armed effector cell. The first signal is delivered by T-cell receptor (TCR)/CD3 complex, and the second signal is delivered by costimulatory molecules such as CD28. To explore whether the function of CD28 was

associated with costimulatory signal, the location of CD3 protein was examined by IHC during early pregnancy (Figure 5).

Blocking CD28 Reduced the Number of ISs in Mice

Whether *CD28* would impact embryo implantation, A-ODNs of *CD28* which to block *CD28* were injected into mice uteri on D3.5 of pregnancy. Another 3 groups were injected with reverse A-ODNs of *CD28*, ddH₂O, and nothing as controls. To examine whether the injection was successful, expression of *CD28* mRNA and protein was detected by real-time qPCR and IHC. As the half-life of ODNs is between 24 and 48 hours,¹⁷ we examined the mRNA and protein of *CD28* at 24 hours (D4.5) and 48 hours (D5.5) after injection. The expression level of *CD28* mRNA and protein of A-ODNs group was significantly lower than that of the other 3 groups (Figure 6A-C). But there was no difference among the *CD28* reverse A-ODNs group, water group, and control group (Figure 6A-C).

We then recorded the number of ISs on D5.5 and D7 to observe whether embryo could successfully implant and develop further. We found that the number of ISs in the *CD28* A-ODNs group was markedly less than that of the other 3 groups on D5.5 and D7 (Figure 7A-C). The number of ISs showed no difference among *CD28* reverse A-ODNs group, water group, and control group (Figure 7A-C). Additionally, there was no obvious difference in the morphology and size

Figure 6. Effect of antisense ODNs on the CD28 expression in the uterus. A, The mRNA level of CD28 on D4.5 (24 hours after injection) examined by qPCR. B, The mRNA levels of CD28 on D5.5 (48 hours after injection) examined by qPCR. *, $P < .05$ and **, $P < .01$. Data are shown as mean $\pm$ standard deviation (SD). C, Expression of CD28 examined by immunohistochemistry (IHC). Reverse A-ODNs, A-ODNs, water, and control stand for the uteri injected with CD28 reverse antisense oligodeoxynucleotides, CD28 antisense oligodeoxynucleotides, ddH₂O, and nothing, respectively (200 $\times$). Bar = 100 μ m. le indicates luminal epithelium; ge, glandular epithelium; s, stroma; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction.

of the embryos in each group (Figure 7A and B). These results suggested that blocking CD28 could reduce the number of ISS rather than influence the development of implanted embryos.

Discussion

A number of literatures have indicated that T cells play a role in embryo implantation.^{8,18,19} It is now widely believed that naive CD4⁺ T cells require 2 distinct signals to proliferate and subsequently differentiate into the armed effector cells that mediate adaptive immunity.¹¹ T-cell receptor/CD3 complex can recognize major histocompatibility complexII and deliver the first signal. The second signal is termed a costimulatory signal.

After the first signal is delivered, T cell will proliferate and differentiate into an armed effector cell when its receptor receives a costimulatory signal. B7/CD28 family is the most classic T-cell costimulatory molecules. In this study, we found an organized expression of CD28 in mice uteri during early pregnancy. Our data showed that the expression of CD28 mRNA in endometria upregulated obviously and reached a peak on D5 of pregnancy and reduced thereafter. The expression of CD28 mRNA increased gradually from D1 to D4 in the stroma of uterus and reached maximum on D5, thereafter decreased on D6 and D7. The expression of CD28 protein was consistent with CD28 mRNA. The expression pattern of CD3 protein examined by IHC was similar to that of CD28. These data

Figure 7. Effect of antisense ODNs on the number of implantation sites. Representative uteri of mice on D5.5 and D7 are shown in (A) and (B). The red arrows mean implantation sites stained by trypan blue and the black arrows mean ovaries. C, displays the number of implantation sites on D5.5 and D7 individually (n = 10 in each group). Data are shown as mean ± standard deviation (SD). ** means a significance with *P* < .01.

indicate that CD28 might participate in embryo implantation through delivering costimulatory signal.

To further explore whether the expression of CD28 is induced by competent blastocysts, the expression pattern of CD28 in early pseudopregnancy was tested. Different from normal pregnancy, there was no significant peak of *CD28* mRNA and CD28 protein from Pd1 to Pd7. When there are blastocysts in the uterus, embryo implantation can be initiated on D5.²⁰ In the absence of blastocysts in the uterus of pseudo-pregnant mice, embryo implantation can't be initiated. Therefore, the expression of CD28 may be affected by implanted blastocysts.

In nonpregnant mouse uteri, intraepithelial T cells have an important role in prevention of intrauterine infection and pathogen resistance.^{21,22} On D0, the expression of CD28 at epithelium may be due to this reason. To the maternal endometrium, embryo is an allograft that may be rejected by maternal immune system. Embryo implantation contains 3 stages named apposition, adhesion, and invasion.²³ We found that there was a relatively low expression of CD28 at ISs on D5, compared to that at IISs. As the mouse uterus is receptive for blastocyst implantation between days 4 and 5 of gestation,³ we consider that a successful embryo adhesion may be relevant to the reduction in CD28 delivered costimulatory signal.

Pregnancy has been considered as a model of successfully controlled tissue invasion, where trophoblast cells infiltrate the maternal decidua without being rejected or without destroying the tissue. In choriocarcinoma and hydatidiform mole, a

dysregulation of invasive trophoblast cells was present.²⁴ Abundant expression of CD28 in decidua during D5 to D7 of pregnancy implied that recruitment of immune cells in stroma may prevent excessive embryo invasion and lead to a successful pregnancy. CD28 expressed in stroma of uteri in pseudo-pregnant mice is much less than that in pregnant mice. These results support the speculation that recruitment of immune cells in stroma prevent excessive embryo invasion. Previous studies have indicated that during early pregnancy, there are numerous immune cells recruitment such as nature killer T cells, regulatory T cells, and dendritic cell.^{8,25,26} Additionally, endometrium from women with unexplained infertility contained significantly fewer CD8⁺ T cells than fertile controls.²⁷ Blocking *CD28* by injection of the uterine horn with *CD28* A-ODNs, the number of ISs showed an obvious reduction. So, the immune function of CD28 in stroma of uteri is beneficial for controlling excessive invasion. However, over activation and consequent attack of maternal immune cells to the fetus are postulated to be important causes of some pregnancy complications, such as recurrent abortion and preeclampsia.^{28–30} An abnormal increase in CD28 was also found in human early pregnancy miscarriage.³¹ Therefore, we consider that an appropriate costimulatory signal is important for successful embryo implantation.

In conclusion, we consider that the expression pattern of CD28 in mouse endometria has a spatial temporal characteristic during early pregnancy and pseudopregnancy. The high level of CD28 in mouse endometria during the implantation window suggests that CD28 may be involved in the process

of embryo implantation. We speculate the role may be relevant to costimulatory signal delivered by CD28 in stroma. However, the detailed mechanism by which CD28 fulfills its role in implantation needs further exploration.

Acknowledgments

The authors would like to thank all the members in our research group for their technical support, and Lianju Shen for her writing assistance.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship and/or publication of this article: This work was supported by the Natural Science Foundation of China (grant number: NSFC, 31071278).

References

- Latham KE, Garrels JI, Chang C, Solter D. Analysis of embryonic mouse development: construction of a high-resolution, two-dimensional gel protein database. *Appl Theor Electrophor*. 1992;2(6):163-170.
- Psychoyos A. From Lataste to the “window of implantation”: 100 years of fascinating discoveries [in French]. *Contracept Fertil Sex*. 1993;21(4):333-338.
- Paria BC, Huet-Hudson YM, Dey SK. Blastocyst's state of activity determines the “window” of implantation in the receptive mouse uterus. *Proc Natl Acad Sci USA*. 1993;90(21):10159-10162.
- Yoshioka S, Fujiwara H, Nakayama T, Kosaka K, Mori T, Fujii S. Intrauterine administration of autologous peripheral blood mononuclear cells promotes implantation rates in patients with repeated failure of IVF-embryo transfer. *Hum Reprod*. 2006;21(12):3290-3294.
- Takabatake K, Fujiwara H, Goto Y, et al. Intravenous administration of splenocytes in early pregnancy changes the implantation window in mice. *Hum Reprod*. 1997;12(3):583-585.
- Takabatake K, Fujiwara H, Goto Y, et al. Splenocytes in early pregnancy promote embryo implantation by regulating endometrial differentiation in mice. *Hum Reprod*. 1997;12(10):2102-2107.
- Tian X, Zhang L, Zhang X. Lymphocytes and perforin expression in endometrium during the menstrual cycle. *Chin Med J (Engl)*. 2000;113(10):930-933.
- Erlebacher A. Immunology of the maternal-fetal interface. *Annu Rev Immunol*. 2013;31:387-411.
- Sanguanserm Sri D, Pongcharoen S. Pregnancy immunology: decidual immune cells. *Asian Pac J Allergy Immunol*. 2008; 26(2-3):171-181.
- Fu B, Li X, Sun R, et al. Natural killer cells promote immune tolerance by regulating inflammatory TH17 cells at the human maternal-fetal interface. *Proc Natl Acad Sci USA*. 2013;110(3):E231-E240.
- Janeway CA Jr, Bottomly K. Signals and signs for lymphocyte responses. *Cell*. 1994;76(2):275-285.
- Ling V, Munroe RC, Murphy EA, Gray GS. Embryonic stem cells and embryoid bodies express lymphocyte costimulatory molecules. *Exp Cell Res*. 1998;241(1):55-65.
- Chen X, He J, Ding Y, et al. The role of MTOR in mouse uterus during embryo implantation. *Reproduction*. 2009; 138(2):351-356.
- Ma XH, Hu SJ, Ni H, et al. Serial analysis of gene expression in mouse uterus at the implantation site. *J Biol Chem*. 2006;281(14): 9351-9360.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods*. 2001;25(4):402-408.
- Li S, Chen X, Ding Y, Liu X, Wang Y, He J. Expression of translationally controlled tumor protein (TCTP) in the uterus of mice of early pregnancy and its possible significance during embryo implantation. *Hum Reprod*. 2011;26(11):2972-2980.
- Wagner RW. Gene inhibition using antisense oligodeoxynucleotides. *Nature*. 1994;372(6504):333-335.
- Klentzeris LD, Bulmer JN, Warren A, Morrison L, Li TC, Cooke ID. Endometrial lymphoid tissue in the timed endometrial biopsy: morphometric and immunohistochemical aspects. *Am J Obstet Gynecol*. 1992;167(3):667-674.
- Vassiliadou N, Bulmer JN. Quantitative analysis of T lymphocyte subsets in pregnant and nonpregnant human endometrium. *Biol Reprod*. 1996;55(5):1017-1022.
- Giudice LC. Potential biochemical markers of uterine receptivity. *Hum Reprod*. 1999;14(suppl 2):3-16.
- Pollard JK, Mitchell MD. Intrauterine infection and the effects of inflammatory mediators on prostaglandin production by myometrial cells from pregnant women. *Am J Obstet Gynecol*. 1996; 174(2):682-686.
- Matsuoka T, Matsubara T, Katayama K, Takeda K, Koga M, Furukawa S. Increase of cord blood cytokine-producing T cells in intrauterine infection. *Pediatr Int*. 2001;43(5):453-457.
- Enders AC. Anatomical aspects of implantation. *J Reprod Fertil Suppl*. 1976;(25):1-15.
- Knoeller S, Lim E, Aleta L, Hertwig K, Dudenhausen JW, Arck PC. Distribution of immunocompetent cells in decidua of controlled and uncontrolled (choriocarcinoma/hydatidiform mole) trophoblast invasion. *Am J Reprod Immunol*. 2003;50(1):41-47.
- Loke YW, King A. Immunology of implantation. *Baillieres Best Pract Res Clin Obstet Gynaecol*. 2000;14(5):827-837.
- Silasi M, Mor G. Decidual stromal cells as regulators of T-cell access to the maternal-fetal interface. *Am J Reprod Immunol*. 2012;68(4):279-281.
- Klentzeris LD, Bulmer JN, Warren MA, Morrison L, Li TC, Cooke ID. Lymphoid tissue in the endometrium of women with unexplained infertility: morphometric and immunohistochemical aspects. *Hum Reprod*. 1994;9(4):646-652.
- Laird SM, Tuckerman EM, Cork BA, Linjawi S, Blakemore AI, Li TC. A review of immune cells and molecules in women with recurrent miscarriage. *Hum Reprod Update*. 2003;9(2):163-174.
- Dekker GA, Sibai BM. The immunology of preeclampsia. *Semin Perinatol*. 1999;23(1):24-33.
- Matthiesen L, Berg G, Ernerudh J, Ekerfelt C, Jonsson Y, Sharma S. Immunology of preeclampsia. *Chem Immunol Allergy*. 2005; 89:49-61.
- Jin LP, Fan DX, Zhang T, Guo PF, Li DJ. The costimulatory signal upregulation is associated with Th1 bias at the maternal-fetal interface in human miscarriage. *Am J Reprod Immunol*. 2011;66(4):270-278.