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Progesterone Promotes HOXa-10 Expression in Mouse During Embryo Implantation Period

Di Wu, Guanglei Li, Ruiyi Lin, Changchun Li and Shuhong Zhao
Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction,
Ministry of Education, Key Laboratory of Swine Genetics and Breeding,
Ministry of Agriculture, Huazhong Agricultural University,
430070 Wuhan, Hubei, P.R. China

Abstract: *HOXa-10* (HomeoboxA10) gene plays an important role in embryo implantation and development regulated by Progesterone (P4) which is secreted mainly through corpus luteum cells to regulate the embryo development. The study mainly investigated the effect of progesterone on the expression of HOXa-10 in the endometrium during the embryo implantation. Kunming mice were injected progesterone (2 or 4 mg/each per day), respectively from early pregnant D1 (the 1st day of pregnancy) to D4 (the 4th day of pregnancy) or D6 (the 6th day of pregnancy) by intraperitoneal injection. The endometrium samples of early pregnant D4, D5 (the 5th day of pregnance) and D6, from 3 groups were collected with each group containing 3 mice. Real-time fluorescence Quantitative PCR (QPCR) was used to analyze the expression of the mouse *HOXa-10* gene. The result demonstrated that the expression level of HOXa-10 was increased at pregnant D4 and there was a peak at pregnant D5 then it began to decrease at pregnant D6. Besides, the expression level of HOXa-10 was dependent on the dose of progesterone.

Key words: HOXa-10, Progesterone (P4), QPCR, mouse, injection

INTRODUCTION

Litter size is an important economic trait in animal husbandry. It is important to improve the litter size for the development of livestock farming. Embryo implantation is a key essential process for embryo development. The successful implantation needs the synchronism of embryonic development and the uterine differentiating into the receptive state (Carson *et al*., 2000). There are mainly three ways of embryo implantation in different animal species: centric, eccentric and interstitial, the mouse belongs to the way of rapid eccentric implantation (Lee and DeMayo, 2004). But the detailed regulation mechanism of the implantation is still not understood.

Hox genes are developmentally regulated transcription factors which share a highly conserved sequence element encoding a 61 amino acid helix turn helix DNA binding domain. *Hoxa-10* gene is one key member of Homeobox genes of the Abdominal B (AbdB) family (Benson *et al*., 1995), it is expressed widely in the uteri of humans (Taylor *et al*., 1998) primates (Godbole *et al*., 2007), mice (Taylor *et al*., 1997; Zhao *et al*., 2006) and bitches (Guo *et al*., 2009). Earlier study have indicated that in Ishikawa cells progesterone increased the HOXa-10 expression through progesterone receptors (Liu *et al*., 2007). In canine uterus the expression level of HOXa-10 is in a high level on day 20 of pregnancy in the glandular epithelium when embryo implants and it gradually declines from day 23 and reaches a low level on day 28 (Guo *et al*., 2009). The non-expression of HOXa-10 will lead to a small litter size because of failure of implantation (Bagot *et al*., 2000). These studies showed that HOXa-10 is required to regulate the expression of important factors that direct embryonic development (Taylor *et al*., 1998).

Progesterone (P4) regulates expression of many genes which are necessary for the endometrial remodeling and embryo-maternal communications. It up-regulates the HOXa-10 expression in cultured human cells (Taylor *et al*., 1998). P4 can also highly modulate HOXa-10 expression in primates (Godbole *et al*., 2007) and bitches (Guo *et al*., 2009) *in vivo* or *in vitro*. Besides, the expression level of HOXa-10 is dependent on the dose of physiological concentrations (0.1-1000 nM) of P4 in stromal cells (Taylor *et al*., 1998). When the expression level of HOXa-10 at D7 day after pregnancy is higher than the normal pregnancy, the blastocyst nidation rate will

Corresponding Author: Shuhong Zhao, Department of Animal Science, Huazhong Agricultural University, Wuhan, 430070 Hubei, P.R. China

increase (Zhou *et al*., 2008). Earlier studies focused on the effects of P4 to the HOXa-10 using isolated cells (Taylor *et al*., 1998; Blitek *et al*., 2010). Few studies have investigated their relationship in individual level and in different time points after pregnancy. Therefore, researchers further investigated the effect of P4 based on different dose to HOXa-10 at different time surrounding the implantation period in this study. The results help to provide potential novel methods for increasing the litter size in mammals.

MATERIALS AND METHODS

Animal and tissue preparation: A total of 45 female and 10 male Kunming mice weight 26-28 μg were purchased from the disease prevention and control centers of Hubei province. The male and female were 1:1 co-caged, the 1st day of pregnancy was defined as D1 when the female vaginal plug was observed to calculate the days of pregnancy. Pregnant mice were divided into nine groups randomly (denoted as D4-control, D4-2 mg, D4-4 mg; D5-control of D5-2 mg, D5-4 mg and D6-control, D6-2 mg, D6-4 mg), each group contains five mice. Mice in D4-D6 group were injected by progesterone from 1st day of pregnancy (D1) to the 4th day (D4), 5th day (D5) and 6th day (D6) of pregnancy. The control, 2 and 4 mg represent the normal control group which did not receive injection, abdomen injection of progesterone 2 mg/each 1 day and abdomen injection of progesterone 4 mg/each 1 day, respectively. Mice were housed in groups of four in filter-top cages and maintained on 12:12 h light-dark cycles with *ad libitum* access to water and a standard chow diet. The mice were killed by cervical dislocation under sterile conditions and endometrium was collected, stored at -80°C.

RNA extraction and first strand cDNA synthesis: Total RNAs from the mouse tissues were isolated using TRIzol reagent (Invitrogen) and treated with deoxyribonuclease I (Fermentas, Vilnius and Lithuania). About 1.2% agarose gel was used to check the quality of the RNA by formaldehyde-denaturing gel electrophoresis. The RNA concentration was examined using NanoDropTM 1000 Spectrophotometer (Thermo Fisher Scientific Inc., USA). Total RNAs were subsequently used as a template for first-strand cDNA synthesis using a revertaid first stand cDNA Synthesis kit (Promega) in a 50 μL reaction.

Reactions were incubated for 1 h at 42°C and heated to 75°C for 10 min. Approximately 1 μL of the completed reactions was used as a template for PCR.

Table 1: Primer pairs used for QPCR amplification of mouse *HOX1-10* gene

Gene	Sequence (5'-3')	Product size (bp)	Annealing temp. (°C)
HOXa-10	Forward: 5'-ATATCGGCTGAGGAGCTGTCT-3'	143	58
	Reverse: 5'-CGTGTAAGGGCAGCGTTT-3'		
β-actin	Forward: 5'-GAGACCTTCAACACCCCAGC-3'	263	58
	Reverse: 5'-CCCTTTAGCACGCACTGTA-3'		

Real-time fluorescence quantitative PCR analysis of the differential expression of the *HOXa-10* gene in the endometrium of P4 treated mouse: Real-time fluorescence Quantitative PCR (QPCR) was performed in triplicate on a Roche LightCycler® 480 Real-Time system (Roche Inc.) in 25 mL total volume reactions using SYBR Green PCR Master Mix (Toyobo Inc.) to detect the HOXa-10 differential expression in the mouse endometrium of different pregnant stages and injected dose of progesterone.

The primer sequences used for real-time PCR were shown in Table 1 which were processed by initial denaturation at 95°C for 3 min then 95°C for 30 sec, 58°C for 30 sec and 72°C for 15 sec for 45 cycles. Melting curves were obtained by increasing the temperature from 56-95°C at 0.5°C sec^{-1} for 15 sec. Data were analyzed by the Comparative Critical Threshold Method (Livak and Schmittgen, 2001) in which normalized by the amount of β-actin mRNA and expressed relative to the corresponding value in control mouse. The expression levels of gene between groups were compared using Student's t-test and a $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

***HOXa-10* gene expression in mouse endometrium at D4 of pregnancy:** QPCR results showed the endometrial expression of HOXa-10 is significantly increased ($p<0.05$) when the dose of P4 was 4 mg/each 1 day in D4 (D4-4 mg) group compared with the control group. However, no significant differences were observed of the expression of HOXa-10 between D4-2 mg and the control group ($p>0.05$) or D4-2 mg and D4-4 mg group. These results suggested that the expression level of HOXa-10 was dependent on the dose of P4 at D4.

The time of mouse embryo implantation is around the 4.5 days of pregnancy (D4.5) and previous study has shown that the expression of the HOXa-10 reached a peak at D4 in endometrium of normal pregnant mouse. The result indicated that the expression of mouse HOXa-10 in endometrium at the time of implantation will be up-regulated with abdomen injection of P4 (Fig. 1).

***HOXa-10* gene expression in mouse endometrium at D5 of pregnancy:** The results showed the endometrial

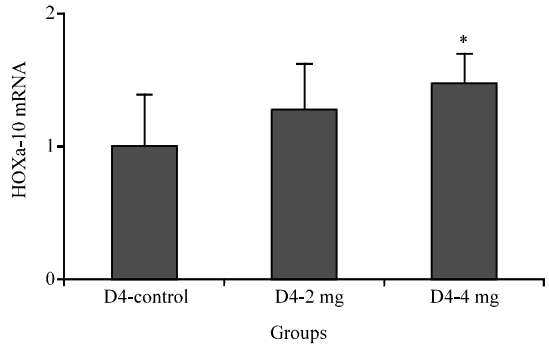

Fig. 1: The mRNA levels of mouse *HOXa-10* gene in endometrium induced by P4 at D4 of pregnancy; D4 represents the days of pregnancy and P4 injection; control represents the normal control group which did not receive P4 injection; 2 and 4 mg represents the abdomen injection of progesterone 2 and 4 mg/each 1 day, respectively. The data are expressed as mean±SEM (n = 3). Asterisks indicate a statistically significant difference from the activity in the absence of estrogen (*indicates $p<0.05$)

expression of HOXa-10 significantly increased ($p<0.05$) when the dose of P4 was both 2 mg/each 1 day in D5 (D5-2 mg) and 4 mg/each 1 day in D5 (D5-4 mg) group compared with the control group (D5-control). In normal pregnant mice, the contents of endogenous P4 already begins to decrease at D5 and the expression level of HOXa-10 is also reduced. But in the study, the expression of HOXa-10 in endometrium is increased after abdomen injection of P4. However, no significant differences were observed for the expression of HOXa-10 between D5-2 mg and the control group ($p>0.05$) and D5-4 mg group (Fig. 2).

***HOXa-10* gene expression in mouse endometrium at D6 of pregnancy:** The expression of HOXa-10 in mouse endometrium at D6 of pregnancy is shown in Fig. 3. The results showed that there is no significant difference between the three groups at D6 of the expression of HOXa-10 in mouse endometrial.

Successful implantation of embryos needs not only the ability of blastocyst implantation but also the endometrial, receptivity. The two factors independent and related processes need the hormones regulation and the communication of placenta and uterus (Norwitz *et al*., 2001). Earlier studies have proved that the portable embryos could wait the uterus to implant while the accepting uterus could not wait the embryos. The accepting state of uterus owned a short time to permit the embryos to implant (Goto *et al*., 1993). The main period is at the 4th day after insemination. The ability to accept the

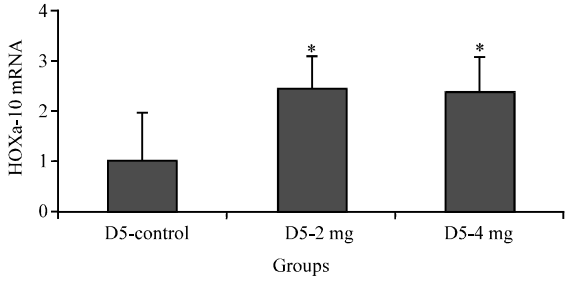

Fig. 2: The mRNA levels of mouse *HOXa-10* gene in endometrium induced by P4 at D5 of pregnancy; D5 represents the days of pregnancy and P4 injection; control represents the normal control group which did not receive P4 injection; 2 and 4 mg represents the abdomen injection of progesterone 2 and 4 mg/each 1 day, respectively. The data are expressed as mean±SEM (n = 3). Asterisks indicate a statistically significant difference from the activity in the absence of estrogen (*indicates $p<0.05$)

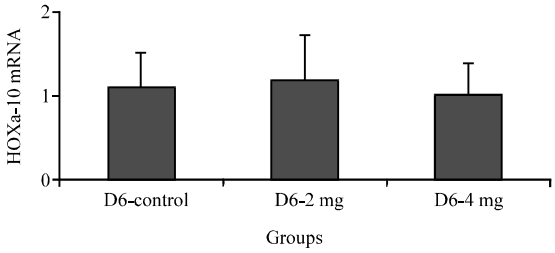

Fig. 3: The mRNA levels of mouse *HOXa-10* gene in endometrium induced by P4 at D6 of pregnancy; D6 represents the days of pregnancy and P4 injection; control represents the normal control group which did not receive P4 injection; 2 and 4 mg represents the abdomen injection of progesterone 2 and 4 mg/each 1 day, respectively. The data are expressed as mean±SEM (n = 3). Asterisks indicate a statistically significant difference from the activity in the absence of estrogen (*indicates $p<0.05$)

embryos to implant declines at 5 day and it will disappear at 6 day (Paria *et al*., 1993; Song *et al*., 2002). The disorder of the implantation period will lead to the failure of the embryo development (Wilcox *et al*., 1999; Song *et al*., 2002; Ye *et al*., 2005). The embryo implantation happens in the dioestrus phase in mouse where it is strictly regulated by hormones (Finn and Martin, 1974). With more studies focused on the mechanisms of implantation, many factors were proved to participate the implantation. HOXa-10 is an important transcription factor for

implantation (Yao *et al*., 2003). The expression of HOXa-10 reaches the highest level in the implantation window and its expression level is in concordance with the progesterone (Taylor *et al*., 1998). There are many studies on the effect of progesterone on HOXa-10 have many while most of them focused on the isolated cells. The study is aimed to determine the best adaptive dose of P4 to maintain the high expression level of HOXa-10 thus embryos could implant to the uterus at 4 or 5 days pregnancy.

CONCLUSION

The study shows that the expression level of HOXa-10 is depended on the dose of the P4 and the pregnant time. Besides, the injection of P4 before the 6 days was useful to increase the expression of HOXa-10, it will be trashy after 6 days so researchers can make the dose at 4 mg or more for per mouse before the 6 days to improve the expression of HOXa-10. Zhou have reported that the expression of HOXa-10 at D7 of pregnancy was decreased to the similar level to control (Zhou *et al*., 2008). Researchers supposed that there are some other factors which can reduce the effect of P4 to the expression of HOXa-10 at D6-D7. Researchers can also apply this method in other livestock breeding. However, further studies should be conducted to confirm that the injection of P4 can contribute to increase the litter size.

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