

## RESEARCH

# Implantation failure in rats with subclinical hypothyroidism is associated with LIF/STAT3 signaling

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## Abstract

**Background:** Pregnant women with subclinical hypothyroidism are associated with an increased risk of spontaneous abortion. This study aims to investigate the mechanisms underlying the effects of maternal subclinical hypothyroidism during early pregnancy on abortion in the uterus, focusing upon the LIF/STAT3 signaling pathway.

**Methods:** One hundred five Wistar rats were randomly divided into three groups (35 rats in each group): control (CON) group, subclinical hypothyroidism (SCH) group and overt hypothyroidism (OH) group. We examined the weight of rat uteri, rat placenta and embryos. We also determined the number of implantation sites and the embryo absorption rates. The protein and mRNA expressions of TSHR, TR- $\alpha$ , TR- $\beta$ , LIFR, gp130, JAK1, p-STAT3 and STAT3 were measured by immunohistochemical staining, real-time PCR and Western blotting.

**Results:** The weights of rat uteri, rat placenta and embryos were significantly reduced in the SCH and OH groups. The number of implantation sites was significantly decreased in the SCH and OH groups, while embryo absorption rates were significantly increased. The mRNA and protein expressions of TSHR were upregulated in the SCH and OH groups, while TR- $\alpha$  and TR- $\beta$  showed no difference when compared between the three groups. The expression levels of LIFR, gp130, JAK1 and p-STAT3 were significantly higher in the SCH and OH groups.

**Conclusions:** Clinical and subclinical hypothyroidism during early pregnancy might cause adverse pregnancy outcomes. Implantation failure in rats with subclinical hypothyroidism was associated with abnormal LIF/STAT3 signaling.

## Key Words

- ▶ mild hypothyroidism
- ▶ abortion
- ▶ leukemia inhibitory factor
- ▶ implantation window
- ▶ endometrial receptivity

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## Introduction

Subclinical hypothyroidism (SCH), characterized by elevated levels of thyroid-stimulating hormone (TSH) with total thyroxine (TT4) within the normal reference range, is the most common type of thyroid disorder in pregnancy (1). A previous large cohort study in America used trimester-specific references to classify the definition

of hypothyroidism in pregnancy and found that 15.5% of pregnant women had increased TSH during pregnancy, of which 2.4% were overt hypothyroidism (OH) and 97.6% were SCH (2).

Clinical studies have shown that pregnant women with OH or SCH are likely to have an increased risk of

pregnancy complications such as spontaneous abortion. Although SCH leads to a lower incidence of pregnancy complication compared to OH, the risk of spontaneous abortion still increases with the prevalence of SCH (3, 4). Negro *et al.* demonstrated that women with TSH levels between 2.5 and 5.0 mIU/L in the first trimester and those who are negative to thyroid antibody are associated with a higher rate of spontaneous pregnancy loss as compared with women with TSH levels lower than 2.5 mIU/L (5). However, the mechanisms underlying miscarriage caused by SCH are still unclear.

Leukemia inhibitory factor (LIF) is known to play a vital role in the progression of blastocysts to implantation. In humans, LIF regulates the receptivity of the uterus by controlling blastocyst implantation via the promotion of proliferation and differentiation (6). LIF can also regulate extravillous trophoblast (EVT) invasion by inducing the phosphorylation of STAT3 and plays an important role in the implantation of the embryo into the endometrium (7).

For rats, successful implantation only occurs during a short limiting period generally from 4 to 6 days of pregnancy (8). During 12 to 14 days, the occurrence of abortion (in our previous experiments) can be clearly observed. In this study, we used a model of rats with SCH during pregnancy and observed the implantation site and abortion rate on day 5 and day 13 of pregnancy respectively, focusing particularly on the LIF/STAT3 signaling pathway.

## Materials and methods

### Animals

One hundred five nulliparous female Wistar rats, weighing 180–200 g (virgin 6–7 weeks old), were maintained on a 12:12-h light/darkness cycle and fed normal rat chow *ad libitum*. All animals and experimental procedures were approved by the Animal Care and Use Committee at China Medical University, which complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The rats were randomly assigned to three groups: control group (CON,  $n=35$ ), OH group ( $n=35$ ) and SCH group ( $n=35$ ). The rats were treated by intraperitoneal (i.p.) injection of 3% pentobarbital sodium (0.1 mL/100 g) and placed on an operating table. Rats in the OH and SCH groups were subjected to thyroidectomy, removal of the thyroid gland, to establish a hypothyroid model. Rats in the CON group were given a sham operation

that did not involve removal of the thyroid gland, as described in a previous work (9). After surgery, rats were kept at  $34 \pm 2^\circ\text{C}$  under an electric blanket until they awoke. Four weeks after surgery, rats in the SCH group were injected subcutaneously with L-thyroxine (L-T<sub>4</sub>, Sigma) 1.0  $\mu\text{g}/100\text{g}/\text{day}$  on the back. Rats in the CON and OH groups were injected subcutaneously with physiological saline (50  $\mu\text{L}/100\text{g}/\text{day}$ ) on the back. Calcium lactate (0.1% w/v) was added to the drinking water for all rats after surgery. After 9 days of injections, all rats were mated with normal male rats (male:female=1:2). The day of vaginal plug was confirmed by microscopic observation and designated as E0. We collected tissues on days E0, E5 and E13. We measured the weight of the uterus on days E0 and E5 and the weight of the placenta and embryos on day E13. We also calculated the number of implantation sites (Fig. 2A) and the absorptions (Fig. 2B). We used the ratio of number of absorptions and number of all the embryos (viable and absorbed) represented the absorption rate.

### Measurement of TT4 and TSH

Blood samples obtained from all groups were immediately centrifuged at 13,000  $\times g$  for 13 min and stored at  $-80^\circ\text{C}$  for subsequent measurement of TT4 and serum TSH using a supersensitive chemiluminescence immunoassay (Immulite). All samples were assayed in duplicate. The intra- and interassay coefficients of variation were below 10% and the sensitivity for TT4 was 1  $\mu\text{g}/\text{dL}$ . The results below this limit of quantification were recorded at 1  $\mu\text{g}/\text{dL}$  for statistical purposes.

### RNA isolation and quantitative real-time PCR

RNA was extracted from rat uteri from each group using TRIzol (Life Tech). First strand of cDNA was synthesized using total RNA and RT-PCR was carried out by TaqMan expression assays, using glyceraldehyde-3-phosphatedehydrogenase (GAPDH) as a control (R&D Systems). The sequences for TSHR, TR- $\alpha$ 1, TR- $\alpha$ 2, TR- $\beta$ , LIFR, Gp130, JAK1 and GAPDH were performed through the ABI PRISM system. Primer sequences are shown in Table 1 and were analyzed with BLAST software on NCBI. Reactions were performed in a total volume of 20  $\mu\text{L}$  and gene expression was determined by SYBR Premix Ex Taq TM II (TaKaRa Biotechnology Co., Ltd.) in accordance with the manufacturer's instructions. Reactions began with a 10 s hot activation of Taq polymerase at  $95^\circ\text{C}$ , followed by 40–45 cycles of amplification in three steps (denaturation at  $95^\circ\text{C}$  for 5 s, 30 s annealing at  $60^\circ\text{C}$

**Table 1** Primer sequences for the real-time PCR.

Gene	Forward primer	Reverse primer
<i>Tshr</i>	CCCTGTCCCTCACTATCTGC	ACTGGTTCCTCGCCTTCAG
<i>Tr-α1</i>	CCACATGAAAGTCGAGTGCC	AAGAGATGGGGTTCCTCCCT
<i>Tr-α2</i>	GGGGAAGGAGAAGGAGCAT	AGGGGTAGGAGGGTGGTCTT
<i>Tr-β</i>	GGTGGCAAGGTTGATCTGGA	CACAGGGCAGCTCACAAAAC
<i>Lifr</i>	CCCACGCAACACAGAATAACA	GGTCAGGAGCCATTTTCAAG
<i>Gp130</i>	ACCACCACCACCTTGACT	GTGCTTCCTCCACCAACATC
<i>Jak1</i>	GGAGGAGCAGAATCCAGACA	TCAACCTCCCAAAGTGACC
<i>Gapdh</i>	GACATGCCGCTGGAGAAAC	AGCCAGGATGCCCTTATG

and 30s extension at 72°C). The mRNA expression was measured as a ratio to GAPDH.

### Immunohistochemistry

Immunohistochemical studies were conducted on uterine tissue. Samples were first embedded in paraffin and then sectioned at a thickness of 5 μm. Tissue samples were then dewaxed and incubated in 0.3% H<sub>2</sub>O<sub>2</sub> for 10 min. For staining, slices were first treated with a heated antigen repair protocol in 0.03 M citrate buffer (pH 6.0) for 40 min at 95°C. All samples were then incubated in 5% bovine serum albumin for 20 min to block nonspecific binding sites after different types of antigen repair. All sections were incubated with rabbit polyclonal antibodies against TSHR (1:500), TR-α (1:100), TR-β (1:300), STAT3 (1:200), and p-STAT3 (1:250). All antibodies were purchased from Abcam and were incubated with sections overnight at 4°C. Slices were then stained with secondary antibody (Santa Cruz Biotechnology). Sections were then stained with DAB (Santa Cruz Biotechnology) and observed under microscope in order to develop positive antibody binding signals. The nuclei of sections were then counterstained with hematoxylin. Finally, sections were dehydrated, rinsed and mounted in neutral gum. All sections were then analyzed at a magnification of ×200. Five visual fields per slide were randomly selected. The area integrated optical density (AIOD) of positive cells in each field was calculated using a Metamorph/DPIO/BX51 morphology image analysis system (Olympus) in order to assess the expression of each target. The results were analyzed using ImagePro Plus software (Bethesda, MD, USA).

### Western blotting

Tissues were lysed in radioimmunoprecipitation (RIPA) buffer and total protein concentration was determined with a bicinchoninic acid (BCA) assay (Beyotime). Then, 20 μg of total protein from each sample was separated by 10% SDS-PAGE and then transferred to PVDF

membranes. Membranes were then washed, blocked and incubated sequentially with specific primary antibodies: rabbit polyclonal antibody LIF (1:1,000), LIFR (1:200); p-STAT3 (1:2,000), ER-α (1:1,000); gp130 (1:1,000); JAK1 (1:5,000) (Abcam) and mouse polyclonal anti-GAPDH (1:3,000; Santa Cruz Biotechnology). Incubation in primary antibodies was followed by incubation with goat anti-rabbit secondary antibody (1:5,000, Santa Cruz Biotechnology). Positive antibody binding reactions were detected by an enhanced chemiluminescence using an ECL system and each experiment was performed in triplicate. The relative densitometry of the band was measured using ImageJ software (Bethesda).

### Statistical analysis

The statistical data are presented as mean ± s.d. SPSS20.0 (IBM) was used for all data analyses. The *t*-test was applied to assess the statistical significance of the differences between paired groups of data, and differences among more than two groups were analyzed using one-way ANOVA. *P* < 0.05 was considered as statistically significant. All artwork was created by using GraphPad Prism (6.0).

## Results

### TT4 and TSH levels in maternal rats

To evaluate maternal thyroid function, blood samples from the CON, SCH and OH groups were evaluated for serum levels of TT4 and TSH. Compared to the CON group, the OH group showed a significant increase in TSH and a reduction in TT4 (Table 2). Serum TSH in the SCH group was significantly higher than in the CON group (*P* < 0.05), but there was no significant difference in TT4 levels between these two groups. The SCH group also had a significantly higher serum level of TT4 than the OH group (*P* < 0.05). Collectively, normal TT4 and higher TSH levels confirmed the successful creation of a rat model of maternal SCH (Table 2). All maternal rats maintained stable levels of TT4 and TSH level when analyzed at E0, E5 and E13 (Table 2).

### Pregnancy outcomes

On E0 and E5, we recorded the weight of rat uteri, it was much lower in the SCH and OH group compared to the control group (*P* < 0.05; Fig. 1A and B). Furthermore, on E13, the weight of rat placenta and embryos were much lower in the SCH and OH groups (*P* < 0.05; Fig. 1C and D).

**Table 2** Serum TSH and TT4 levels in the CON, SCH, and OH groups.

Groups	4 weeks after operation			9 days of injection			E0			E5			E13		
	TSH	TT4	No.	TSH	TT4	No.	TSH	TT4	No.	TSH	TT4	No.	TSH	TT4	No.
CON	0.17 ± 0.04	4.50 ± 0.51	35	0.19 ± 0.08	4.04 ± 0.93	35	0.28 ± 0.25	3.79 ± 0.77	10	0.14 ± 0.20	4.62 ± 1.02	10	0.19 ± 0.05	4.12 ± 0.58	15
SCH	18.20 ± 5.65*	<1.00*	35	4.18 ± 1.09*	3.63 ± 0.71	35	5.79 ± 2.70*	3.66 ± 0.87	10	3.54 ± 1.58*	4.09 ± 1.07	10	4.10 ± 1.10*	3.53 ± 0.60	15
OH	17.6 ± 4.10*	<1.00*	35	15.7 ± 3.04*	<1.00*	35	17.14 ± 3.58*	<1.00*	10	13.33 ± 2.76*	<1.00*	10	11.30 ± 1.63*	<1.00*	15

Data shown as group mean ± s.d.  
\*P < 0.05 vs the same day CON group.

On E5, we observed and recorded the number of implantation sites in each group (Fig. 2A). We found that the number of implantation sites of SCH and OH group were lower than that in the CON group ( $P < 0.05$ ; Fig. 2B). The OH group was the lowest, but there was no significant difference between the SCH and OH groups (Fig. 2B).

On E13, we observed and recorded the number of absorptions in each group (Fig. 2C) and calculated the embryo absorption rates. We found that the embryo absorption rates were significantly higher in the SCH and OH groups ( $P < 0.05$ ; Fig. 2D). The OH group was the highest, but there was no significant difference between the SCH and OH groups (Fig. 2D).

### Uterine TSHR, TR- $\alpha$ 1, TR- $\alpha$ 2 and TR- $\beta$ mRNA expressions

We measured the mRNA expression levels of TSHR, TR- $\alpha$ 1, TR- $\alpha$ 2 and TR- $\beta$  in uterus of the three groups. On E0 and E5, the SCH and OH groups showed an increased expression of mRNA levels for TSHR compared with the CON group, with the OH group showing the most significant increase on E0 ( $P < 0.05$ ; Fig. 3A and E).

There was no significant difference between these groups in terms of the expression of mRNA levels for TR- $\alpha$ 1 ( $P > 0.05$ ; Fig. 3B and F), TR- $\alpha$ 2 ( $P > 0.05$ ; Fig. 3C and G) and TR- $\beta$  ( $P > 0.05$ ; Fig. 3D and H) on E0 and E5.

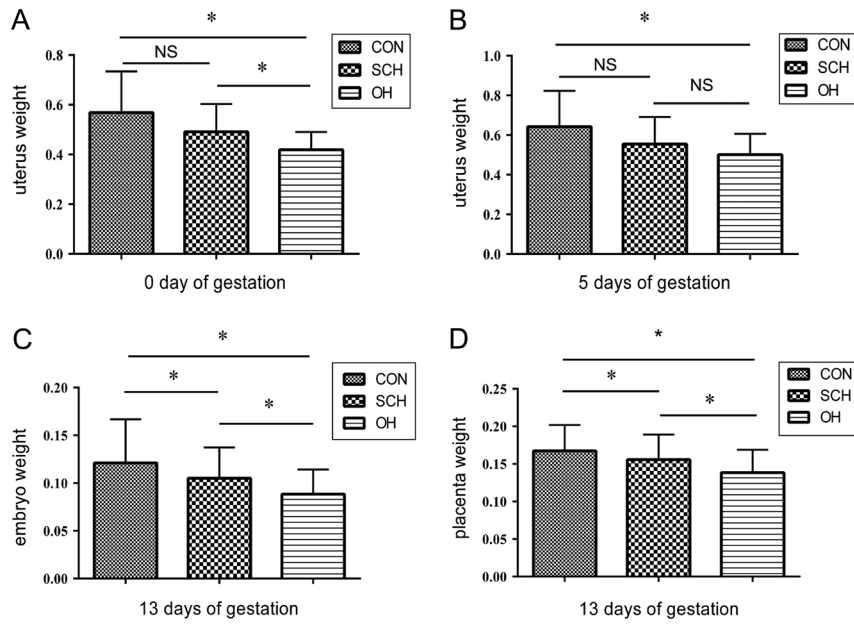
### Immunohistochemical staining of TSHR, TR- $\alpha$ and TR- $\beta$ in the uterus

Immunohistochemical analysis detected expression of TSHR, TR- $\alpha$  and TR- $\beta$  on E0 and E5 (Figs 4A and 5A, C); AOID levels of TSHR in the SCH and OH groups were significantly higher than those in the CON group ( $P < 0.05$ ; Fig. 4B), although there was no significant difference in TR- $\alpha$  and TR- $\beta$  between the three groups ( $P > 0.05$ ; Fig. 5B and D).

### Uterine expression of LIFR, gp130 and JAK1 mRNA

The mRNA levels of LIFR, gp130 and JAK1 were detected on E0 and E5 by real-time PCR. Data showed that on E0, the mRNA levels of these three targets were significantly higher in the SCH and OH groups than in the controls ( $P < 0.05$ ; Fig. 6A, C and E). There was no significant difference between the three groups on E5 ( $P > 0.05$ ; Fig. 6A, C and E).

Furthermore, from E0 to E5, these three indicators showed a general tendency to increase in the control



**Figure 1** (A and B) Uterine weight of pregnant rats from the CON, SCH and OH groups at 0 and 5 days of gestation ( $*P < 0.05$ ). (C and D) Weight of embryos and placenta in pregnant rats from the CON, SCH and OH groups at 13 days of gestation ( $*P < 0.05$ ). NS, non-significant.

group ( $P < 0.05$ ; Fig. 6B, D and F), although no significant difference was detected in the SCH and OH groups ( $P > 0.05$ ; Fig. 6B, D and F).

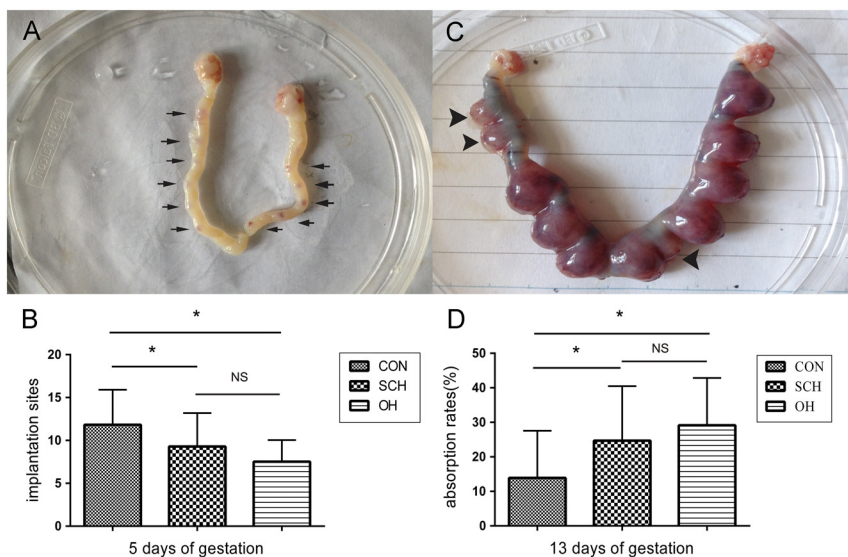
From E0 to E5, these five proteins showed a general tendency to increase in the control group ( $P < 0.05$ ; Fig. 7B (ii), C (ii), D (ii), E (ii) and F (ii)).

### Levels of LIF, LIFR, gp130, JAK1 and p-STAT3 protein in the uterus

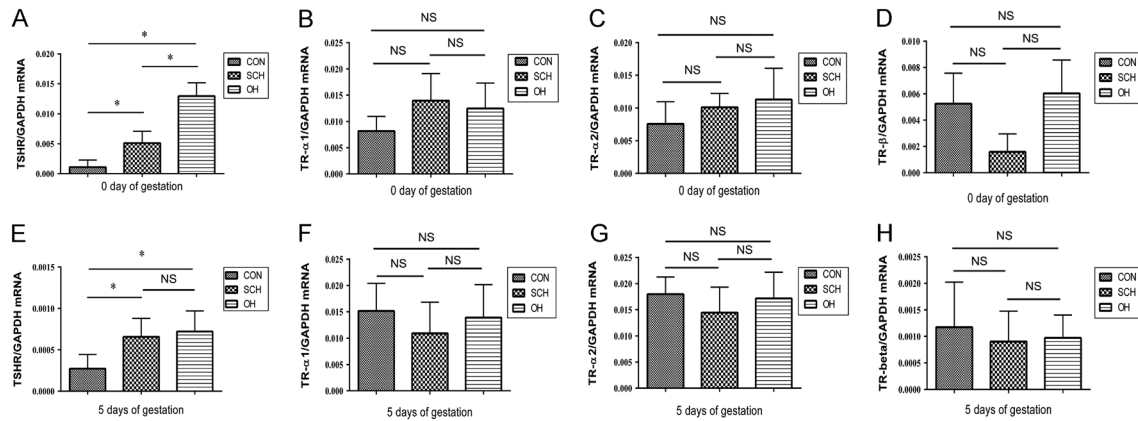
The protein levels of LIF, LIFR, gp130, JAK1 and p-STAT3 were examined by Western blotting (Fig. 7A). Data showed that on E0, the protein levels in the SCH and OH groups were significantly higher than the controls ( $P < 0.05$ ; Fig. 7B (i), C (i), D (i), E (i) and F (i)) and that these five proteins showed no difference between the three groups on E5 ( $P > 0.05$ ; Fig. 7B (ii), C (ii), D (ii), E (ii) and F (ii)).

### Discussion

The interaction between embryo implantation and a receptive uterine environment is a vital aspect of successful embryo implantation (10, 11, 12). A range of cytokines and growth factors act as either autocrine and paracrine factors, with both temporal- and spatial-specificity, in order to regulate uterine receptivity (13, 14). Uterine implantation is classified into three phases:



**Figure 2** (A) The arrows point to the implantation sites of pregnant rats on 5 days of gestation. (B) Implantation sites of pregnant rats from the CON, SCH and OH groups at 5 days of gestation ( $*P < 0.05$ ). (C) The arrows point to the embryos absorbed on 13 days of gestation. (D) Embryo absorption rates from the CON, SCH and OH groups at 13 days of gestation ( $*P < 0.05$ ). NS, non-significant.



**Figure 3**

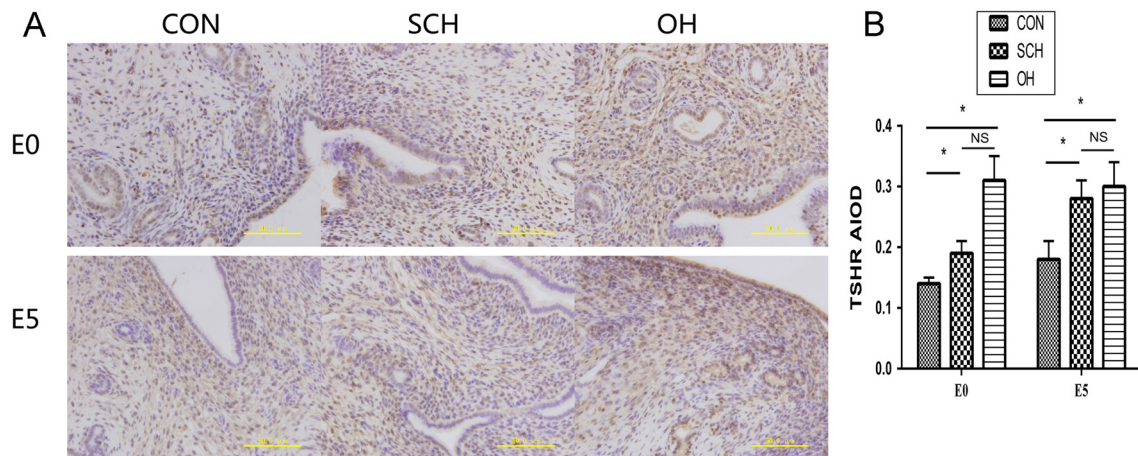
TSHR (A and E), TR-α1 (B and F), TR-α2 (C and G) and TR-β (D and H) (mean ± s.d.) mRNA expression in the uterus from the CON, SCH and OH groups at 0 and 5 days of gestation (\* $P < 0.05$ ). NS, non-significant.

pre-receptive (1–3 days after fertilization), receptive (day 4 after fertilization) and non-receptive phases (day 5 after fertilization) (15, 16). Embryo implantation can only occur during the receptive phase, and the process cannot be completed in the other two phases.

Thyroid hormones play an important role in embryo implantation and the early stages of development. In this study, we used a model of subclinical hypothyroidism in pregnant rats via the complete surgical removal of the thyroid and subcutaneous injection of  $L-T_4$  (9). The results of serological tests in rat models indicated that this method was feasible and reproducible. The uterine tissues of pregnant rats were collected on E0 and E5, and the placentas and embryos were also collected on E13. We found that on E0 and E5, the weight of the uteri

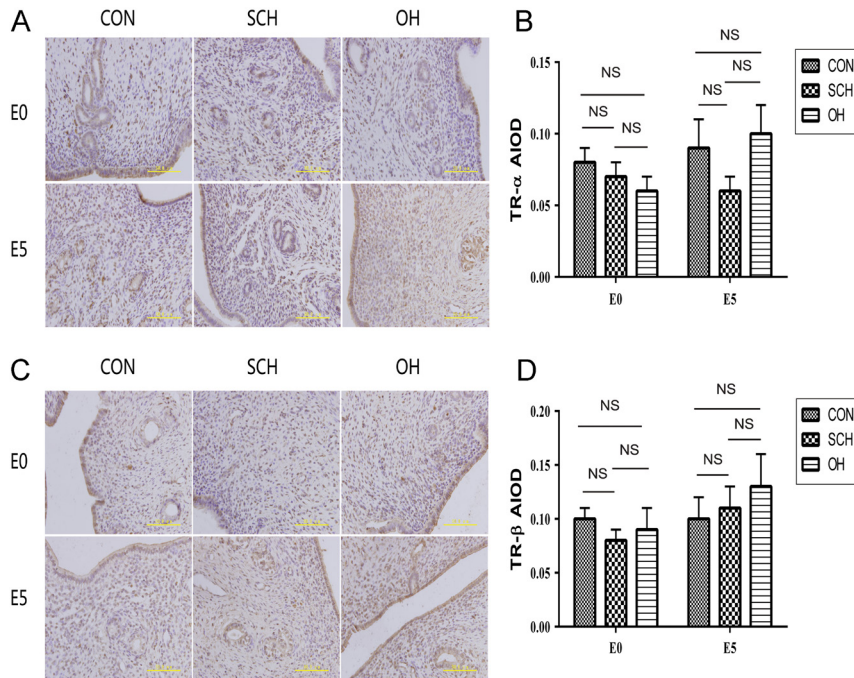
were significantly decreased in the SCH and OH groups compared to the controls. On E13, the weight of the placentas and embryos were significantly reduced in SCH and OH groups. Moreover, the number of implantation sites in the SCH and OH groups were significantly lower than the controls, while embryo absorption rates were significantly increased in the SCH and OH groups. These results suggested that hypothyroidism in pregnant rats, even mild hypothyroidism, had adverse effects on pregnancy outcomes.

The thyroid hormone receptor (TR) hypotype, TR $\alpha$  and TR $\beta$ , bind to T3 and regulate TSH-related gene expression. Previous studies have reported that TR and the TSH receptor (TSHR) play a vital role during the implantation window and show a wide range of



**Figure 4**

TSHR immunohistochemical staining of uteri from the CON, SCH and OH groups at 0 and 5 days of gestation. (A) Immunohistochemical images of TSHR expression (streptavidin-biotin-peroxidase, Harris hematoxylin, scale bar = 50  $\mu$ m). (B) Increased AOID levels of TSHR in uteri from the SCH and OH groups compared with the CON group at 0 and 5 days of gestation (\* $P < 0.05$ ). NS, non-significant.

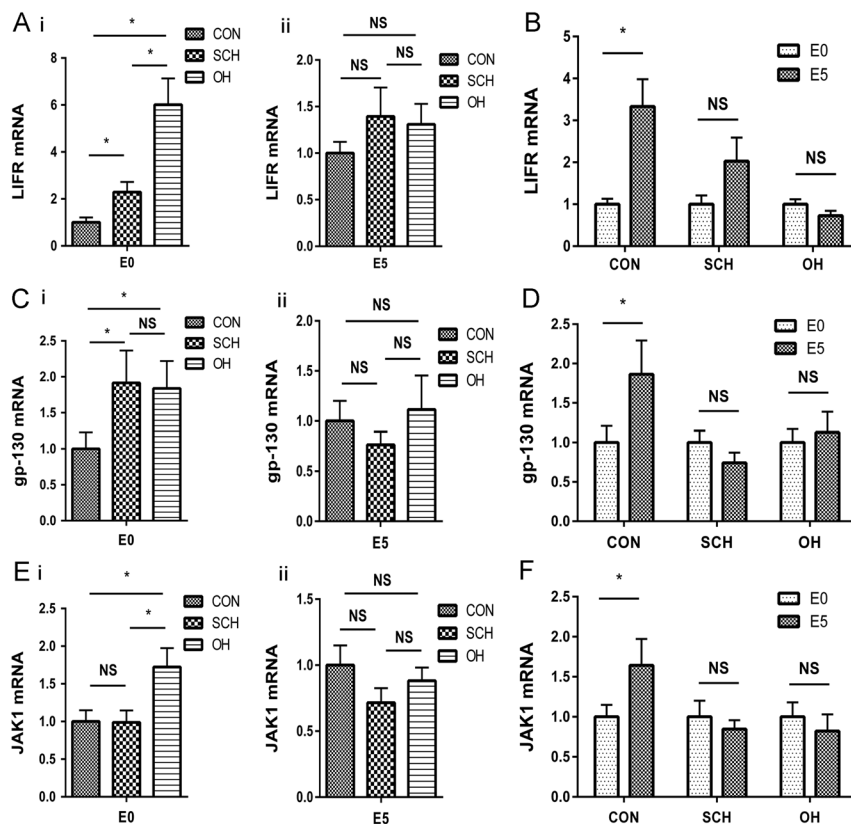


**Figure 5**

TR-α, and TR-β immunohistochemical staining of uteri from the CON, SCH and OH groups at 0 and 5 days of gestation. (A and C) Immunohistochemical images of TR-α and TR-β expression (streptavidin-biotin-peroxidase, Harris hematoxylin, scale bar = 50 μm). (B and D) AOID levels of TR-α and TR-β in uteri from the SCH and OH groups compared with the CON group at 0 and 5 days of gestation (no significant difference). NS, non-significant.

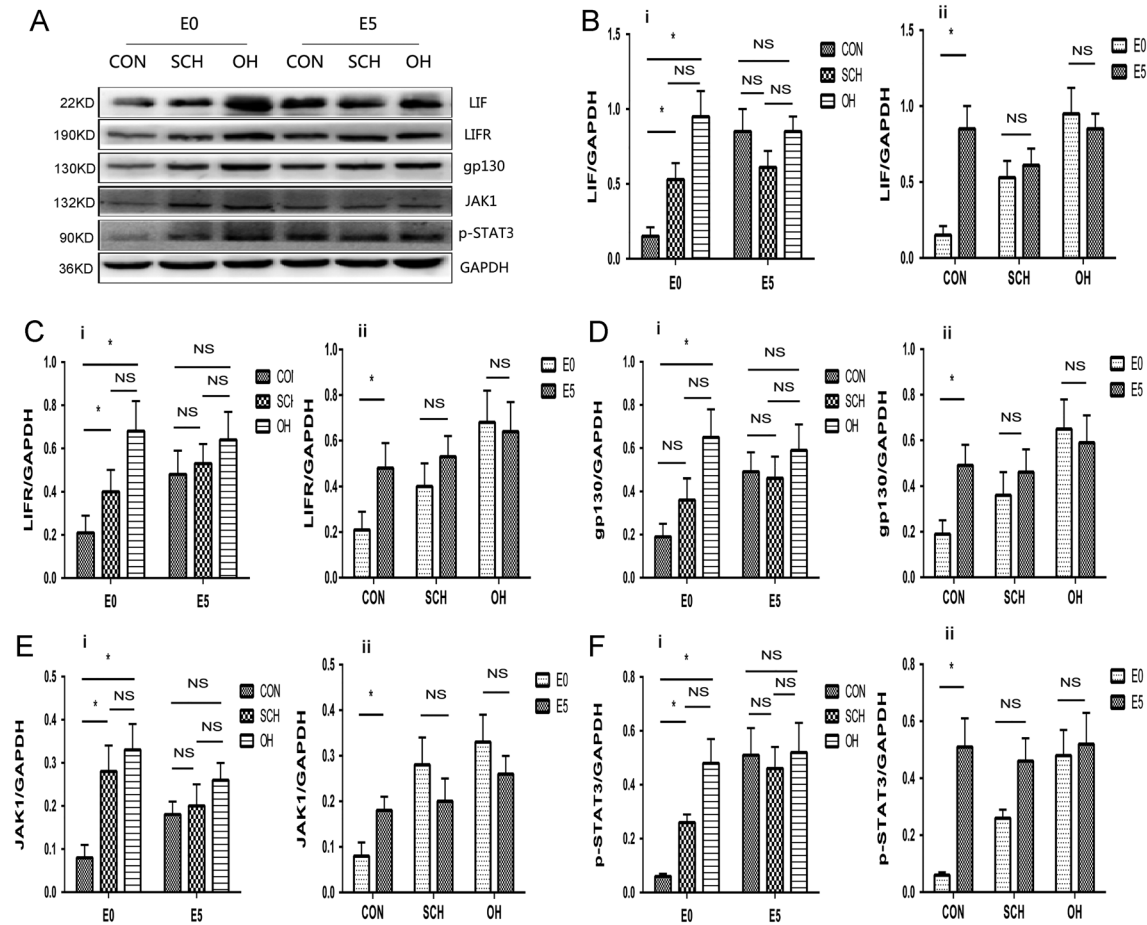
expressions in the maternal fetal unit, thus confirming that TH and TSH in the vicinity of the endometrium and fetus may directly or indirectly regulate the process of embryo implantation (17, 18). Stewart *et al.* suggested

that abnormal menstruation and increased miscarriage are likely to be due to the direct effects of TSH on the uterus in women with abnormal thyroid function (19). In this study, we found that the mRNA and AOID levels



**Figure 6**

LIFR (A and B), gp130 (C and D) and JAK1 (E and F) (mean ± s.d.) mRNA expression in uteri from the CON, SCH and OH groups at 0 and 5 days of gestation (\* $P < 0.05$ ). NS, non-significant.



**Figure 7**

(A) Protein expression of LIF, LIFR, gp130, JAK1 and p-STAT3. LIF (B), LIFR (C), gp130 (D), JAK1 (E) and p-STAT3 (F) protein levels in uteri from the CON, SCH and OH groups at 0 and 5 days of gestation (\* $P < 0.05$ ). NS, non-significant.

of TSHR in the SCH and OH groups were significantly higher than those in the controls, while TR- $\alpha$  and TR- $\beta$  showed no significant difference when compared across the three groups. This is consistent with the results of Stewart *et al.* (19).

LIF is a vital cytokine expressed in human endometrial cells and is known to play a role in the process of implantation (20, 21). Many studies have confirmed that the abnormal expression of LIF is related to abnormal embryo implantation (22, 23). LIF combines with the LIF receptor complex (composed of LIFR and gp130) distributed on the surface of luminal epithelial cells to form a stable LIF-LIFR-gp130 complex (24). This complex can activate JAK1 enzymes, resulting in the phosphorylation of the intracellular domain, results in the recruitment and phosphorylation of the latent signal transducer and activator of transcription 3 (STAT3). In a number of animal studies, disruption of the JAK-STAT

pathway, using a variety of models, ultimately resulted in the failure of blastocyst implantation (25, 26).

It has been demonstrated that TSH can stimulate LIF expression in the thyroid of monkeys (27). An *in vitro* study measured the effects of TSH on the LIF signaling pathway in cultured samples of endometrium (17) and showed that TSH dramatically increased the expression of LIF in stromal cells. Moreover, TSH can also promote the mRNA expression of LIFR, which indicated the participation of TSH in the mediation of LIF signaling (17). In this study, we examined the expression of the LIF-STAT3 pathway in each of our treatment groups. Our results showed that elevated serum TSH upregulated expression of the LIF-STAT3 pathway prior to the uterine receptivity phase, thus promoting the upregulation of LIF-STAT3 in the CON group, but not in the SCH and OH groups during the implantation window. Thus, an abnormal LIF/STAT3 pathway in uterine tissue may be related to abnormal



patterns of embryo implantation in rats in the OH and SCH groups. This supports the hypothesis that elevated TSH may affect the LIF/STAT3 pathway, rendering the blastocyst and uterus in a 'non-synchronous' state, and eventually leading to implantation failure (28).

In conclusion, high levels of TSH exerted influence over the LIF/STAT3 signaling pathway and ultimately led to implantation failure. This mechanism may participate in adverse pregnancy outcomes in women with clinical and subclinical thyroid dysfunction.

#### 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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