


# Interleukin-6 regulates expression of *Fos* and *Jun* genes to affect the development of mouse preimplantation embryos

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## Abstract

**Aim:** We investigated whether recombinant mouse interleukin-6 (IL-6) affects the development of preimplantation embryos and induces the Janus kinase-signal transducers and activators of transcription (JAK–STAT) signaling pathway by binding IL-6 signal transducer (IL-6st) and regulating *Fos* and *Jun* gene expression, thereby accounting for the negative effect of superovulation on embryo development.

**Methods:** We compared rates of blastocyst formation from embryos cultured with different concentrations of IL-6 or/and anti-interleukin 6 receptor antibody (anti-IL-6RAb) in superovulated experimental and normal control groups. IL-6 expression in preimplantation embryos was determined by immunofluorescence identification. *Fos*, *Jun* and IL-6st messenger RNA expression was detected by PCR and microarray experiments.

**Results:** Rates of blastocyst formation significantly decreased in superovulated embryos, whether or not they were incubated in 0.1, 1, 25 or 50 pg/mL IL-6, ( $P < 0.01$ ) compared to embryos from naturally ovulated controls, whereas incubation in 5 and 10 pg/mL IL-6 reversed the negative effects of superovulation. The addition of anti-IL-6RAB to naturally ovulated embryos reduced blastocyst rates to those of superovulated embryos. Gene chip analysis indicated that the JAK–STAT signaling pathway contained differentially expressed *IL-6*, *IL-6st*, *Jun* and *Fos* genes. Both anti-IL-6RAB or ovarian stimulation downregulated *IL-6st*, *Jun*, and *Fos* messenger RNA expression, but expression of the same three genes increased in 5 pg/mL IL-6.

**Conclusion:** Ovarian stimulation negatively impacts the development of preimplantation embryos by reducing IL-6 release. IL-6 affects the rate of development of zygotes to blastocyst by regulating *IL-6st*, *Fos* and *Jun* expression in the JAK–STAT signaling pathway.

**Key words:** preimplantation, embryo, *Fos*, IL-6, *Jun*, mouse, ovarian stimulation.

## Introduction

Assisted reproductive technology (ART) has been an effective infertility treatment for nearly three decades; however, the efficiency of ART is still low.<sup>1–3</sup> Improving ART efficiency is a problem for obstetricians and gynecologists; therefore, many clinics offer the transfer of a single blastocyst, which resembles the

physiological process, to improve the efficiency of clinical pregnancy and to avoid multiple gestations.<sup>4–6</sup> As a result of ovarian stimulation, more than one mature egg can be obtained in each *in vitro* fertilization (IVF) cycle.<sup>7</sup> The critical step for successful embryo transfer then becomes how to choose the best quality single embryo from the numerous selectable embryos.<sup>8</sup> In recent years, morphological evaluation

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has been the most commonly used method to select embryos, but it has been limited by its subjectivity and inability to identify aneuploid embryos.<sup>9,10</sup> Pre-implantation genetic screening is an invasive procedure and the approach can be applicable to single-gene diseases in which the defect has been identified.<sup>11</sup> Hence, it is desirable to develop a non-invasive, objective and quantitative biomarker approach to choose the best embryo, thereby improving the clinical pregnancy rate.

In our previous report, we demonstrated that ovarian stimulation retarded post-implantation development and caused the differential expression of 92 genes in mouse blastocysts.<sup>12</sup> In light of the theory that detection of the differential expression of secreted proteins in culture medium may help the development of a diagnostic approach to identify the 'best' embryo for transfer, we chose, *IL-6* from the differentially expressed genes, which encodes a secreted protein; *IL-6* is also functionally associated with embryonic development. Further study has demonstrated that ovarian stimulation results in the downregulated expression of *IL-6* messenger RNA (mRNA) by mouse blastocysts and reduced *IL-6* secretion from both mouse and human preimplantation embryos.<sup>13</sup> We found that the higher the level of *IL-6* protein present in the blastocyst culture medium, the more blastocysts formed. In this study, the effect of exogenous *IL-6* on preimplantation mouse embryo development was evaluated. The relationship between *IL-6* and *Fos*, *Jun* and *IL-6st* expression in the JAK-STAT signaling pathway was also evaluated.

## Methods

### Ethics statement

Animal experiments were carried out in strict accordance with the recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Committee on the Ethics of Animal Experiments of the Academy of Military Medical Sciences (reference number: 2013M542519) approved the study protocol on March 5, 2014. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

### Animal treatment and zygote collection

Female ICR (CD-1) mice (aged 4–5 weeks) and male ICR mice (aged 7–14 weeks) were provided by the

Center for Experimental Animals, the Academy of Military Medical Sciences, and Peking University Health Science Center, and housed in a specific pathogen-free facility with a 12 h light/12 h dark photo period, at a temperature of  $23 \pm 3^\circ\text{C}$  and a relative humidity of  $44 \pm 2\%$ . Mice were sacrificed by cervical dislocation for the collection of embryos. The donor mice were divided into: natural ovulation control (N) and superovulated (S) groups. Pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) were used for superovulation. Female ICR mice were superovulated by intraperitoneal injection of 5 IU PMSG in 0.2 mL 0.9% NaCl, followed by intraperitoneal injection of 5 IU hCG in 0.2 mL 0.9% NaCl 48–50 h later. The control group was injected with the vehicle at the appropriate times. Donor females were mated with fertile males. On the following morning, the presence of a vaginal plug indicated successful mating, and the time point was designated day 0.5 days post coitum (dpc). The next day was 1.5 dpc, followed by 2.5 and 3.5 dpc. The investigation of gene expression patterns was carried out on blastocysts by microarray analysis and PCR for the control and superovulated groups, with three biological replicates in each group.

### Preparation of different concentrations of interleukin-6 (IL-6) culture medium

To prepare the different concentrations of *IL-6* media, we first prepared 0.1% bovine serum albumin (BSA) in KSOM (BK) and dissolved 10  $\mu\text{g}$  murine *IL-6* in 100  $\mu\text{L}$  distilled water to make a stock solution of 0.1 mg/mL *IL-6*, which could be stored at  $-20^\circ\text{C}$  for at least one month. We then added 10  $\mu\text{L}$  0.1 mg/mL *IL-6* into 990  $\mu\text{L}$  BK to give 100 ng/mL *IL-6*, and then diluted 100  $\mu\text{L}$  100 ng/mL *IL-6* with the 900  $\mu\text{L}$  BK. We used the 10 ng/mL *IL-6*, and prepared from it the 100, 10, 1 and 0.1 pg/mL *IL-6* KSOM media. One day before the test, 20  $\mu\text{L}$  droplets of culture media under oil were prepared in a culture dish and then placed at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  environment.

### Treatment of zygotes with *IL-6* or *IL-6* receptor antibody

Zygotes from naturally ovulated and superovulated mice were collected under a microscope on day 0.5 dpc. In the *IL-6* addition experiments, according to different concentrations of *IL-6* added to *in vitro* culture media, the superovulated zygotes were divided into superovulation control (0 pg/mL *IL-6*), 0.1, 1, 5, 10, 25 and 50 pg/mL *IL-6* groups; ICR mice

zygotes in a natural ovulation cycle were used as the control. In the anti-IL-6RAb addition experiments, according to 10 ng/mL anti-IL-6RAb with or without different concentrations of IL-6 added to *in vitro* culture media, the zygotes were divided into N + anti-IL-6RAb, S, 1 pg/mL IL-6 + anti-IL-6RAb, 5 pg/mL IL-6 + anti-IL-6RAb, 10 pg/mL IL-6 + anti-IL-6RAb and 25 pg/mL IL-6 + anti-IL-6RAb groups; ICR mice zygotes in a natural ovulation cycle were used as the control. Four zygotes were each cultured in a 20  $\mu$ L droplet of KSOM media under oil at 37°C in a 5% CO<sub>2</sub> environment until the embryos reached the blastocyst stage. Culture media were not changed over the entire culture period. Photographs of zygotes, 2-cell, 4-cell, 8-cell and blastocyst stages were taken under the microscope during the *in vitro* development of embryos and the zygote and blastocyst numbers in each group were recorded to determine the rates of blastocyst formation. Three biological replicates were used for each group. In addition, 420 blastocysts in three biological replicates from each 0 pg/mL (N and S groups), 5 pg/mL IL-6 S and anti-IL-6RAb + N group were prepared for the PCR experiment, with 35 blastocysts in each replicate.

#### Immunofluorescence identification

For immunofluorescence identification of IL-6 in preimplantation embryos, zygote, 2-cell, 4-cell, 8-cell and blastocyst stage embryos were collected and fixed with 4% paraformaldehyde for 30 min. After being rinsed with phosphate-buffered saline (PBS), different-stage embryos were permeabilized with 0.5% Triton X-100 for 1 h and then blocked with 2% BSA for 1 h. After washing with PBS, they were then incubated overnight at 4°C with primary rabbit anti-mouse IL-6 antibody (1:100), followed by goat anti-rabbit Alexa Fluor 488 secondary antibody (diluted 1:200) for 1 h at room temperature. Cell nuclei were counterstained with Hoechst 33 324 for 15 min at room temperature in the dark. The negative control embryos were incubated with mouse immunoglobulin G (1:100). No fluorescence signals were detected in the control. Embryos were imaged using a laser-scanning confocal microscope.

#### RNA extraction

For microarray analysis, total RNA was extracted from pools of 150 blastocysts in three replicates for each group (50 blastocysts in each replicate) using a TRIzol Reagent kit according to the manufacturer's instructions. We pipetted the 50 blastocysts into a

35 mm dish and carefully removed the KSOM culture media surrounding the blastocysts, then added 1 mL of TRIzol Reagent at room temperature to effectively homogenize the blastocysts. The homogenized blastocysts could be stored in the 35 mm dish at room temperature for several hours before being used for the next procedure or being transferred to a new tube to be stored at -70°C for at least one month. After incubating the homogenized blastocysts in polypropylene microcentrifuge tubes for 5 min at room temperature, 0.2 mL of chloroform per 1 mL of TRIzol was added, the tubes were shaken vigorously by hand for 15 s and the samples were incubated for 2–3 min at room temperature before being centrifuged at 12 000  $\times$  g for 15 min at 4°C. After removing RNA into a new tube, 5  $\mu$ g of RNase-free glycogen was added as a carrier and 0.5 mL of 100% isopropanol per 1 mL of TRIzol was added to the RNA aqueous phase. The sample was then incubated at room temperature for 10 min, the tube centrifuged at 12 000  $\times$  g for 10 min at 4°C and the pellet washed with 1 mL of 75% ethanol per 1 mL of TRIzol Reagent. We vortexed the sample briefly and centrifuged the tube at 7500  $\times$  g for 5 min at 4°C. After air-drying the RNA pellet for 6 min, we resuspended it in RNase-free water and incubated it in a water bath at 55–60°C for 10–15 min. Diluted RNA samples were then analyzed spectrophotometrically by the A260/A280 ratio method to determine RNA concentration, yield and purity. Methyl aldehyde degeneration gel electrophoresis was then used to further confirm RNA integrity. The total RNA yield was 80–110 ng/pool.

#### RNA amplification

Eighty nanograms of total RNA was used for linear, two-round amplification by *in vitro* transcription using the MessageAmp™ II aRNA Amplification Kit according to the manufacturer's instructions. Briefly, reverse transcription to synthesize first strand cDNA was primed with the T7 Oligo (dT) Primer provided in the kit to synthesize cDNA containing a T7 promoter sequence, and samples were incubated as follows: 2 h at 42°C, 2 h at 16°C and 14 h at 37°C. The final yield of biotinylated aRNA was 80–120  $\mu$ g. The quality and purity of aRNA were determined by RNA integrity, examined by electrophoresis on 1% formaldehyde denaturing gel. These samples were used for the subsequent hybridization.

### Hybridization and slide processing

A total of 15 µg labeled cRNA was fragmented and hybridized to the Mouse Genome 430 2.0 array, which contains 45 000 probe sets analyzing the expression level of over 39 000 transcripts and variants from over 34 000 well characterized mouse genes. The GeneChip arrays were washed and then stained (streptavidin-phycoerythrin) on an Affymetrix Fluidics Station 450, followed by scanning on a GeneChip Scanner 3000.

### Gene chip analysis

The hybridization data were analyzed using GeneChip operating software (GCOS 1.4). The scanned images were first assessed by visual inspection, then analyzed to generate raw data files saved as CEL files using the default GCOS 1.4 setting. A global scaling procedure was performed to normalize the different arrays using dChip software. Finally, we analyzed the data using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Database for Annotation, Visualization, and Integrated Discovery (DAVID), which classify different functional genes into specific pathways associated with mouse embryo development. Three biological replicates were used for each microarray analysis.

### Real-time PCR

The microarray results were further verified using quantitative real-time PCR analyses of IL-6st, Jun and Fos mRNA expression. Two micrograms of total amplified RNA from blastocysts was reverse-transcribed into complementary DNA using a SuperScript II kit, performed under the following thermal conditions: 65°C for 5 min, 42°C for 2 min, 42°C for 50 min and 70°C for 15 min. All quantitative PCR (qPCR) assays were performed using the QuantiTect SYBR Green PCR kit according to the manufacturer's instructions. Following a 2 min carryover prevention

at 50°C and a 15 min initial activation step at 95°C, samples were amplified by 40 cycles of: 15 s denaturation at 94°C, 30 s annealing at 56°C and 30 s extension at 72°C. The total reaction volume was 50 µL. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase primer sequences were chosen as the internal control for sample RNA normalization. Primer sequences for each gene are shown in Table 1. The 2-Delta Delta cycle threshold relative quantification method was performed to analyze the data from the qPCR experiment, as previously described.<sup>14</sup> Three biological replicates were used for each real-time PCR.

### Statistical analysis

Statistical analysis was performed using SPSS version 17.0. A comparison of the mean blastocyst formation rate was performed using chi-squared tests. To adjust the significance level using the Bonferroni method. A probability of  $P < 0.05$  was used to indicate a significant difference. The relative pathways (KEGG and BioCarta) that were enriched in differentially expressed genes were ranked according to their  $P$  values. Pathways with  $P$  values lower than 0.05 were selected.

## Results

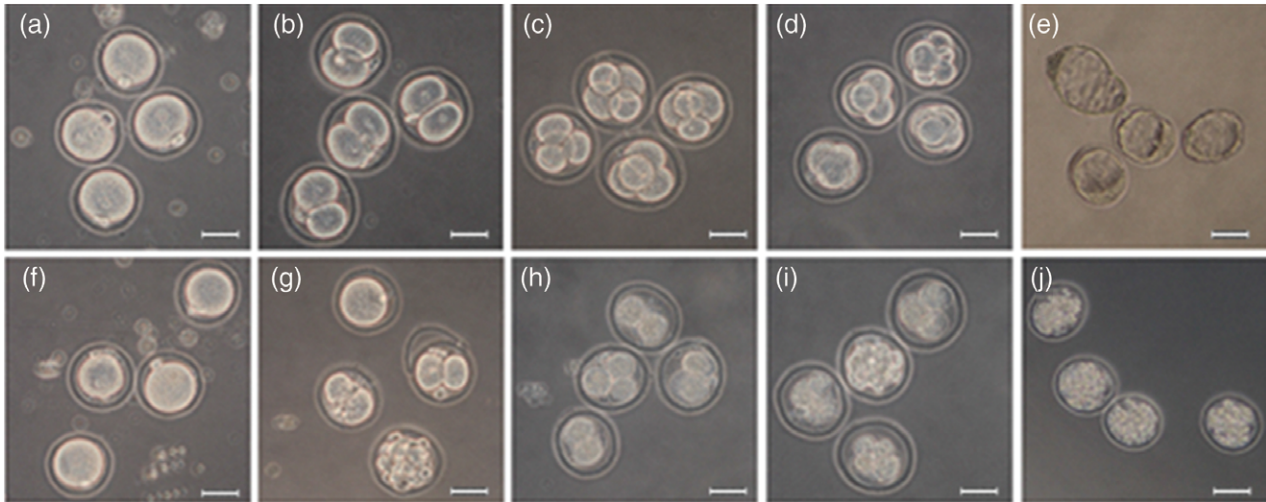
### IL-6 affects the development of preimplantation embryos

To demonstrate the process of zygotes developing into blastocysts in different concentrations of IL-6, we took photos under a microscope to record the embryo morphologies. Representative images of naturally conceived control embryos and 50 pg/mL IL-6 incubated superovulated embryos are shown at zygotic, 2-cell, 4-cell, 8-cell and blastocyst stages (Fig. 1a). The results of this study showed that the rate of blastocyst formation did not significantly differ in the 5 and

**Table 1** Primer sequences for each gene

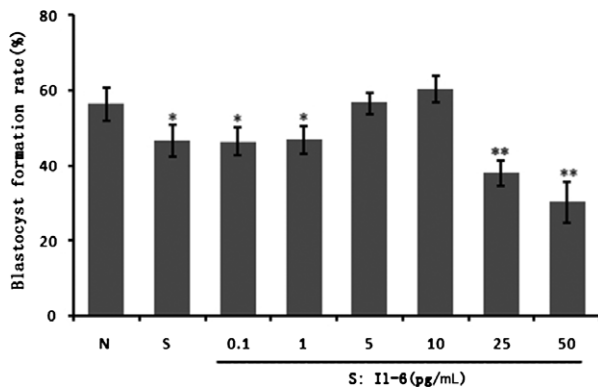
Gene	Primer sequence(5'-3')	Product size (bp)	Accession number
<i>IL-6ST</i>	F: CTGGGTGGAAGCAGAGAATGCCCTTG R: CCGCCAGAAAATCTAGATTT	134	NM_010560
<i>JUN</i>	F: GCTGGAAGAAGGGCTGTTG R: CAGATTCAAAGTTGGAAGGAGAC	196	NM_010591
<i>FOS</i>	F: GTTGTCCTCGGACTTCA R: AATTCCAATAATGAACCCAACA	275	NM_010234
<i>GAPDH</i>	F: GAGATTACTGCTCTGGCTCCTA R: TGGTCCAGGGTTTCTTACTC	182	NM_008084

bp, base pair; F, forward; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; R, reverse.



**Figure 1** The effect of different concentrations of interleukin-6 (IL-6) on the development of preimplantation embryos in naturally ovulated control and superovulated groups. The morphological characteristics of (a) zygotes, (b) 2-cell, (c) 4-cell, (d) 8-cell and (e) blastocyst in the naturally ovulated control group (mice  $n = 20$ ) under the microscope. (f) Zygotes, (g) 2-cell, (h) 4-cell, (i) 8-cell and (j) blastocyst in the 50 pg/mL IL-6 superovulated group (mice  $n = 10$ ). Scale bar = 75  $\mu\text{m}$ .

10 pg/mL IL-6 superovulated groups compared to the naturally ovulated control group (Fig. 2). A significant decrease in the blastocyst formation rate was observed in 0.1, 1, 25 and 50 pg/mL IL-6 experimental groups compared to the normal control group ( $P < 0.05$ ,  $P < 0.01$ ) (Fig. 2).



**Figure 2** Rates of blastocyst formation in naturally ovulated control and superovulated groups treated with different interleukin-6 (IL-6) concentrations (0–50 pg/mL). There was no significant decrease in 0.1 pg/mL and 1 pg/mL IL-6 experimental groups compared with the superovulated group. \*\* $P < 0.01$ , \* $P < 0.05$  indicate significant differences between superovulated and naturally ovulated control groups. Values are expressed as mean  $\pm$  standard deviation.

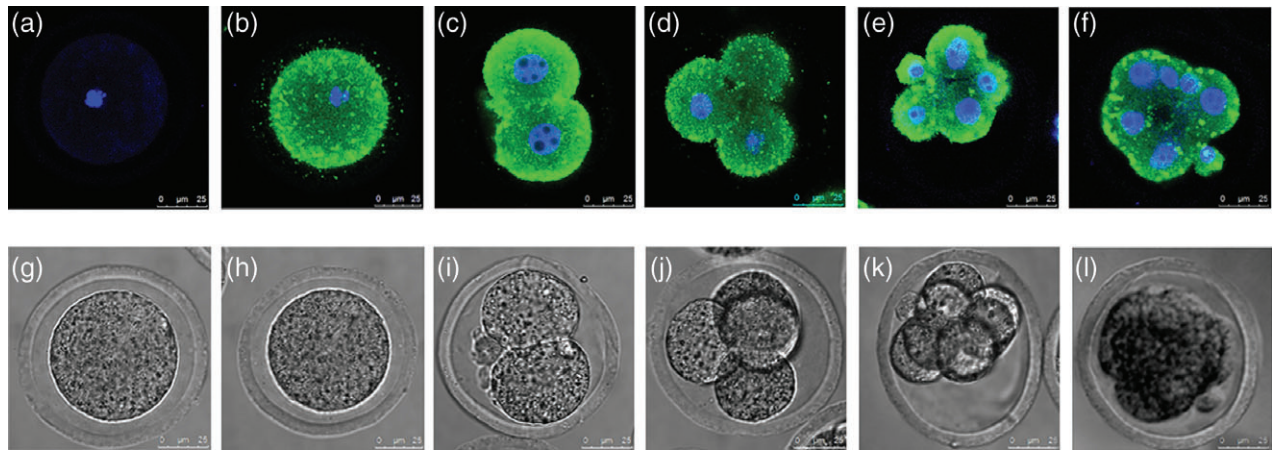
Immunofluorescence identification was used to evaluate *IL-6* gene expression in normal, naturally conceived zygotic, 2-cell, 4-cell, 8-cell and blastocyst stages of mouse embryos. The negative control (Fig. 3a,g) showed no IL-6 expression. Our results showed that IL-6 was expressed and located in the cytoplasm in all stages of preimplantation embryos (Fig. 3b–f,h–l).

#### Anti-IL-6RAb reduces the development rate of preimplantation embryos

The results of this study showed that rates of blastocyst formation decreased significantly in each anti-IL-6RAb group, including the naturally ovulated embryos, compared to the naturally ovulated control group ( $P < 0.01$ ) (Figs 4–5).

#### Pathway analysis of differentially expressed genes and target genes affected by IL-6

DAVID is a set of web-accessible programs developed by the National Institute of Allergy and Infectious Disease.<sup>15,16</sup> It uses the Fisher's exact test to measure gene-enrichment in annotation terms. Based on our previous microarray results,<sup>12</sup> we chose 16 genes upregulated by at least 1.5-fold (superovulated vs naturally conceived groups  $P < 0.01$ ) and 76 genes downregulated by at least 1.5-fold to analyze signaling pathways using DAVID tools and the

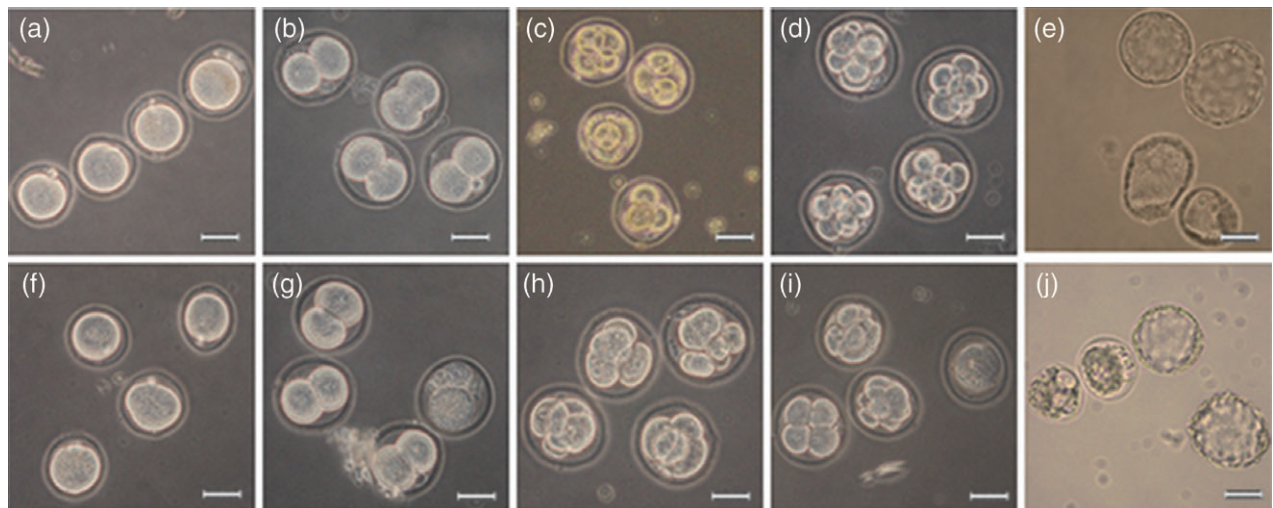


**Figure 3** Immunofluorescence for interleukin-6 (IL-6) protein expression in different stages of preimplantation in naturally ovulated normal control embryos. Two panels were taken under the same confocal laser scanning microscopy: one (a–f) was activated with the corresponding wavelength laser, while the other (g–l) was taken under the bright field without the laser. IL-6 (green); Hoechst33342 (blue). The negative control shows (a) no IL-6 expression and IL-6 expression in (b) zygote, (c) 2-cell, (d) 4-cell, (e) 8-cell and (f) blastocyst. Scale bar = 25 μm.

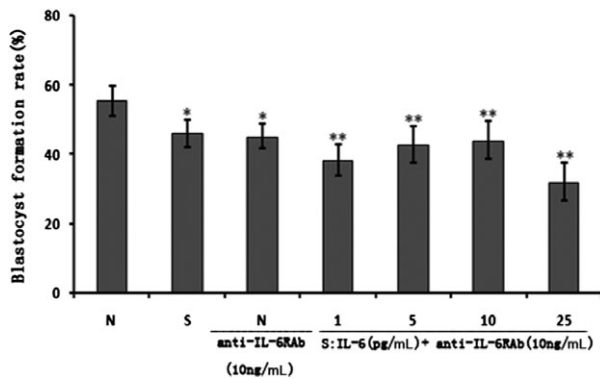
KEGG database. KEGG database results showed that 11 genes were differentially expressed and were associated with mouse embryo development (Table 2) ( $P < 0.01$ ).

The results of expression analysis indicated that IL-6 bound to plasma membrane receptor complexes composed of IL-6 receptor alpha chains and the common signal transducing subunit gp130, which

activates JAK–STAT signaling pathway function. As a result, two phosphorylated Stat3 proteins form a homodimer, which translocates from the cytoplasm to the nucleus where Stat3 activates transcription of the *Fos* and *Jun* genes. Based on microarray results, transcription of the *IL-6st*, *Jun* and *Fos* genes was reduced in blastocysts from superovulated mice. Consistent downregulation of *IL-6st*, *Fos* and *Jun* mRNA was



**Figure 4** The effect of anti-interleukin 6 receptor antibody (anti-IL-6Rab) on the development of preimplantation embryos in naturally ovulated control and superovulated groups. The morphological characteristics of (a) zygotes, (b) 2-cell, (c) 4-cell, (d) 8-cell and (e) blastocyst in the naturally ovulated control group under the microscope. (f) Zygotes, (g) 2-cell, (h) 4-cell, (i) 8-cell and (j) blastocyst in naturally ovulated control + anti-IL-6Rab (10 ng/mL) groups. Scale bar = 75 μm.



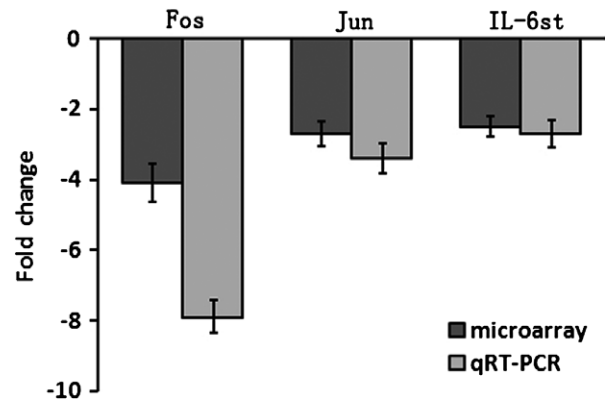
**Figure 5** Rates of blastocyst formation in the naturally ovulated control group (N) with or without treatment with 10 ng/mL anti-interleukin 6 receptor antibody (anti-IL-6RAB) and of the superovulated group (S) with or without treatment with 10 ng/mL anti-IL-6RAB plus different IL-6 concentrations (1–25 pg/mL). \*\* $P < 0.01$ , \* $P < 0.05$ , compared to the naturally ovulated control group. Values expressed as mean  $\pm$  standard deviation.

found by both qPCR analysis and microarray expression (Fig. 6).

To further test the relationship between the expression of *IL-6* and *IL-6st*, *Fos* and *Jun*, we detected *IL-6st*, *Fos* and *Jun* mRNA expression in the presence of *IL-6* or anti-*IL-6RAB* by qRT-PCR. *IL-6st*, *Jun* and *Fos* mRNA expression was downregulated in anti-*IL-6RAB* naturally ovulated blastocysts ( $-3.6$ ,  $-3$ ,  $-8.2$  fold changes, respectively) and the ovarian stimulation group  $-2.7$ ,  $-3.4$ ,  $-7.9$  fold changes, respectively), but upregulated in 5 pg/mL *IL-6* superovulated mice blastocysts (10.6, 4.4, 4.6 fold changes, respectively) compared to the naturally ovulated control blastocysts (Fig. 7).

## Discussion

The aim of this study of zygotes growing to blastocyst was to evaluate the effect of exogenous *IL-6* on preimplantation mouse embryo development and to study the relationship between *IL-6* and *Fos*, *Jun* and *IL-6st*



**Figure 6** Comparison of fold change of *Fos*, *Jun*, and *IL-6st* messenger RNA detected using microarray and quantitative real-time PCR (qRT-PCR) in superovulated mouse blastocysts compared to naturally ovulated control blastocysts. Values expressed as mean  $\pm$  standard deviation.

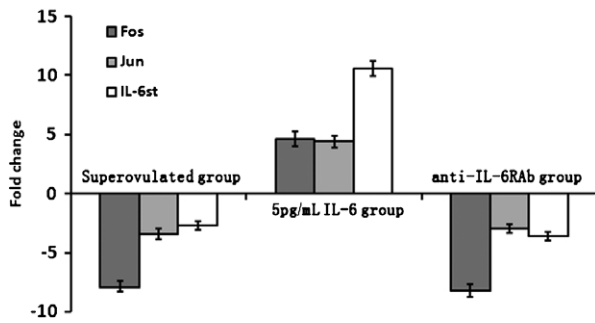
expression in the JAK–STAT signaling pathway. Our results revealed that the rates of blastocyst formation significantly decreased in superovulated embryos and addition of anti-*IL-6RAB* group embryos compared to naturally ovulated control embryos, whereas incubation in 5 and 10 pg/mL *IL-6* reversed the negative effects of superovulation. Immunofluorescence analysis displayed *IL-6* expression in cytoplasm in every stage of the preimplantation embryos. Quantitative PCR analysis and microarray expression confirmed consistent downregulation of *IL-6st*, *Fos* and *Jun* mRNA in the superovulated group. Finally, *Fos*, *Jun* and *IL-6st* mRNA levels were upregulated in the *IL-6* group and deregulated in the *IL-6* anti-*IL-6RAB* group, compared to the control.

Interleukin-6 is known as one of the autocrine cytokines transcribed from the blastocyst stage and plays important roles in proliferation and implantation.<sup>17</sup> *IL-6* mRNA levels increased dramatically during cumulus cell: oocyte complex (COC) expansion, both *in vivo* and *in vitro*. More importantly, when COCs were *in vitro* matured in the presence of *IL-6*, they had much greater embryo transfer efficiency than those without *IL-6* and were comparable to *in vivo* matured

**Table 2** Three highly significant pathways in preimplantation embryos

Pathway name	Total	Gene	<i>P</i>
MAPK signaling pathway	6	<i>Hspb1</i> , <i>FOS</i> , <i>Map4k4</i> , <i>Dusp1</i> , <i>Tgfb2</i> , <i>Gadd45g</i>	0.000
Toll-like receptor signaling pathway	4	<i>FOS</i> , <i>Ly96</i> , <i>Nfkb1a</i> , <i>Il-6</i>	0.000
Jak-STAT signaling pathway	4	<i>Socs3</i> , <i>Ccnd1</i> , <i>IL-6</i> , <i>FOS</i>	0.001

$P < 0.05$  based on Kyoto Encyclopedia of Genes and Genomes database. Jak-STAT, Janus kinase-signal transducers and activators of transcription; MAPK, mitogen-activated protein kinases.



**Figure 7** Comparison of fold change of Fos, Jun, and IL-6 signal transducer (IL-6st) messenger RNA detected using quantitative real-time PCR analysis in superovulated mice blastocysts, 5 pg/mL IL-6 superovulated mice blastocysts and anti-interleukin 6 receptor antibody (anti-IL-6RAb) naturally ovulated blastocysts compared to control naturally ovulated blastocysts. Values expressed as mean  $\pm$  standard deviation.

oocytes.<sup>18</sup> Dominguez *et al.* investigated IL-6 concentrations in sequential media from single blastocysts that subsequently implanted ( $n = 20$ ) versus those that did not implant ( $n = 24$ ), and obtained statistical differences between these two groups (270 pg/ $\mu$ L vs. 1640 pg/ $\mu$ L;  $P < 0.001$ ).<sup>19</sup> In viable embryos, greater IL-6 protein was consumed from the media compared to blastocysts that did not implant. This indicates that IL-6 consumption by the blastocyst could be necessary for either blastocyst development or for the preparation of the implantation process.<sup>19</sup> It has previously been reported that the addition of 10 or 100 ng/mL of recombinant IL-6 to the culture medium did not affect the development of 2-cell stage embryos into blastocysts. However, the total cell number was significantly increased, and apoptosis was reduced in blastocyst stage embryos cultured in the presence of 100 ng/mL of recombinant IL-6.<sup>20</sup> In our study, the addition of 5 and 10 pg/mL of recombinant IL-6 to the culture medium of embryos obtained from superovulated mice favored embryo development from the zygote stage into blastocysts, and there were no significant differences compared to the normal control group. However higher IL-6 concentrations of 25 and 50 pg/mL or lower concentrations of 1 and 0.1 pg/mL were associated with a decline in blastocyst formation rates ( $P < 0.05$ ). After adding anti-IL-6RAB to the culture medium, the rates of blastocyst formation from zygotes *in vitro* decreased significantly compared to the control group ( $P < 0.01$ ), which suggests that the IL-6 plays a role in preimplantation embryo development over a limited concentration range.

There is considerable evidence that IL-6 synthesis is elevated at the time of implantation.<sup>21</sup> Both mRNA and protein levels of IL-6 seem to be hormonally modulated in mouse blastocysts during implantation.<sup>17</sup> The greatest bioactivity of IL-6 was observed on days 5 and 6 of pregnancy in mice at the endometrial trophoblast interphase.<sup>22</sup> In addition, the presence of IL-6 was measured in the conditioned medium of the oocytes, granulosa cells, cumulus cells, one to eight-cell embryos and sperm.<sup>23</sup> IL-6 secretion was detected at all developmental stages of the embryo in this study and was located in the cytoplasm near the cell membrane, indicating that IL-6 may also be involved in the development of preimplantation embryos derived from embryonic secretions. Cytokines of the gp130 family, IL-6 in particular, play a central role in the growth and survival of malignant plasma cells.<sup>24</sup> IL-6 and related cytokines, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin-like cytokine factor 1 - (CLCF1) are all pleiotropic cytokines that share the common receptor IL-6st and exhibit overlapping biological functions.<sup>25</sup> Activation of LIF and IL-11 receptors is essential for embryo attachment and decidualization in mice. Both receptors induce activation of the Stat family of signal transducers via the JAK-STAT pathway.<sup>26</sup> In our previous report, microanalysis identified 92 genes, including *IL-6st*, *Fos* and *Jun* that were differentially expressed in superovulated blastocysts.<sup>12</sup> The results of the present study demonstrate that there are three highly significant pathways associated with development of preimplantation embryos ( $P < 0.05$ ), based on the KEGG database. IL-6st, Jun and Fos take part in the JAK-STAT signaling pathway. After binding of IL-6 to membrane bound IL-6R, the IL-6/IL-6R complex associates with gp130, and then the signal is transduced into the nucleus where Stat3 activates transcription of the *Fos* and *Jun* genes (*Fos* and *Jun* combine to form *AP-1*). IL-6 receptor cDNA encodes a precursor type I transmembrane protein of 460 amino acids (aa) that contains a 19 aa signal sequence, a 345 aa extracellular ligand binding domain, a 21 aa transmembrane region and a 75 aa cytoplasmic segment.<sup>27</sup> Anti-IL-6RAB can bind to a high-affinity IL-6R $\alpha$ , thereby preventing the formation of the IL-6/IL-6R $\alpha$  complex and blocking the binding to gp130, such that the signal cannot be transduced into the cell. Stat3 plays a central role in IL-6-mediated cell proliferation by inhibiting apoptosis in a variety of cell types, which may



regulate cell apoptosis in early mouse development.<sup>20</sup> Apoptosis occurs during the normal development of blastocysts, by removing unnecessary cells.<sup>20</sup> This observation may reflect the ability of IL-6/Stat3 to suppress the apoptotic process at the molecular level, which is an important process for further development in preimplantation embryos.<sup>20</sup> Furthermore, the presence of 5 pg/mL of recombinant IL-6 led to the activation of the JAK–STAT signaling pathway and to increased levels of *AP-1*. This finding verifies that IL-6/IL-6st can regulate *AP-1* expression via the JAK–STAT pathway and plays a role in the regulation of preimplantation embryonic development.

In conclusion, the results of this murine model study indicate that IL-6 may be potentially useful as a non-invasive and quantitative biomarker to evaluate the developmental competence of preimplantation embryos. The effect of IL-6 induction of the JAK–STAT signaling pathway on the development of preimplantation embryos, through binding to the IL-6st and regulating *AP-1* expression, accounts for the negative effect of superovulation on embryo development via the depressed synthesis of IL-6. Measurement of IL-6 gene expression may also help us to identify the 'best' embryo for transfer in order to improve the rate of pregnancy and reduce the rate of multiple pregnancies.

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## Disclosure

The authors have no potential conflicts of interest to disclose.

## Author Contributions

All authors have read and approved the final version of the manuscript.

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