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Supplementary Material

Sealed culture system for supporting mouse preimplantation embryo development *in vitro*

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Experiment design

In order to screen out the most suitable environment for embryo sealed-culture, we selected two different kinds of reference gas mixtures composed of 5% O_2, 5% CO_2, 90% N_2 or 10% O_2, 5% CO_2, 85% N_2 (Longhui, Beijing, China) to pretreat culture medium, and embryos were cultured with three different densities (embryo numbers/n: culture medium volumes/μL) 1:1, 1:2 and 1:4, namely, 200, 100 and 50 of 4-cell embryos were cultured in 200 μL of medium. These elements were combined into 6 sealed-culture groups, marking them simply as follows: the 5% O_2-1:1 group, 5% O_2-1:2 group, 5% O_2-1:4 group, 10% O_2-1:1 group, 10% O_2-1:2 group and 10% O_2-1:4 group. The sealed culture controls were not treated with reference gas and were defined as follows: The No-gas-1:1 group, No-gas-1:2 group, and No-gas-1:4 group. In the general controls, 100, 50 and 25 embryos were loaded into 100 μL of medium drop with mineral oil cover in dishes, respectively, the general controls were defined as follows: the control-1:1 group, control-1:2 group, and control-1:4 group. The sealed PCR tubes were incubated in a 37 °C incubator, and the dishes of general control groups were incubated in an atmosphere of 5% CO_2 at 37 °C.

In this study, the quality of embryos were evaluated by the blastocyst rates, hatching rates, the blastocyst total cell numbers (TCN), apoptosis rates, ROS level and HIF-1α accumulation. For the 4-cell stage embryo sealed-culture, the blastocyst rates were calculated at 60 h and 84 h incubation, and the hatching rates were calculated at 84 h incubation. The TCN were counted at the time point of 60 h and 84 h incubation and cell apoptosis rates were calculated at 84 h incubation. ROS level was detected from 12 h incubation at the first time, and the following detections were performed subsequently every 24 h until there was no difference between the groups. The level of HIF-1α protein was firstly detected in blastocyst in each group of 84 h time point, and was then examined in the group at 60 h time point if accumulation of HIF-1α was detected in the same group at 84 h, similarly, the expression of HIF-1α was evaluated in the group at 36 h time point if HIF-1α accumulation was observed in the same

group at 60 h. For the 2-cell stage embryo sealed-culture, the blastocyst rates and TCN were counted at 72 and 96 h incubation, and the hatching rates were counted at 96 h incubation. The well-developed blastocysts used for embryo transfer were 2-cell stage embryos cultured for 72 h in 5% O_2-1:2 sealed-culture system or in drop.

Once the PCR tubes were opened, they would not be put back to the incubator again. Three replications were performed in each group in each time point of the test mentioned above.

Nuclear counts and cell apoptosis analysis

In this experiment, 20-30 blastocysts were used to count the TCN or detect cell apoptosis in each group. For nuclear counts, embryos were fixed in 4% paraformaldehyde for 1 h and incubated with 10 μg/mL Hoechst 33342 (Beyotime Institute of Biotechnology, Shanghai, China) for 5 min at room temperature, then, the nuclei (at 346 nm excitation) were counted under a fluorescence microscope (Olympus IX71, Tokyo, Japan). For cell apoptosis analysis, embryos were fixed in 4% paraformaldehyde for 1 h and blocked in formaldehyde containing 3% H_2O_2 for 10 min at room temperature. Then, embryos were permeabilized in 0.1% Triton X-100 on ice for 2 min, and the labeling reaction was performed by incubating each sample with 50 μL of TUNEL reaction mixture containing 5 μL terminal deoxynucleotidyl transferase and 45 μL fluorescein-labeled dUTP (Roche, Basel, Switzerland) at 37 °C for 1 h in the dark. Embryos were then counterstained with 10 μg/mL Hoechst 33342 for 5 min at room temperature. After that, the apoptotic cell (at 488 nm excitation) and nuclei (at 346 nm excitation) were counted under a fluorescence microscope (Olympus IX71, Tokyo, Japan).

Detection of the intracellular ROS

Intracellular ROS production was measured by staining with DCFHDA (Sigma, MO, USA). At the 30 min before sealed cultivation ended, 25 μg/mL DCFHDA was added to the culture medium, and then, the PCR tubes were resealed and incubated at 37 °C for 30 min. Afterwards, embryos were counterstained with Hoechst 33342. The DCFH fluorescence was visualized at 504 nm excitation and the nuclei were

visualized at 346 nm excitation under a fluorescence microscope. Three replications were performed in each group in each time point.

Immunohistochemistry

The accumulation of hypoxia-inducible factor 1 alpha (HIF-1α) was detected by immunostaining. All reagents for fixation, wash and blocking were purchased from Beyotime (Beyotime Institute of Biotechnology, Shanghai, China). Embryos were fixed in 4% formaldehyde in PBS (pH 7.4) for 2 h and then blocked in immunostaining blocking buffer (contains Triton X-100) for 1 h at room temperature. Embryos were then incubated with rabbit anti-HIF-1α (1:1000 dilution, Abcam, SF, USA) overnight at 4 °C, followed by peroxidase-conjugated goat anti rabbit IgG antibody for 10 min. The reactions were visualized with DAB. Every step above was followed by 3 washes in PBS. Embryos were dehydrated, mounted on glass slides, and then visualized using an Olympus imaging system. 8-10 morulas (in 36 h 5% O2-1:1 group) or blastocysts (in 60 and 84 h groups) were randomly selected to detect the accumulation of HIF-1α in each assay, three independent assays were performed at different time points in each group.

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted and then reverse transcribed to complementary DNA with a SuperScript III CellsDirect cDNA Synthesis System (Invitrogen, USA). qRT-PCR analysis was used to measure the expression of genes encoding IGF-1, IGF-1R, IGF-2, IGF-2R, EGF, EGFR, TGF-α and GAPDH (as an internal control). The RT-qPCR assay was performed on a Step One Plus Real-Time PCR System (Applied Biosystems; USA) using a SYBR Premix Ex Taq II Kit (TaKaRa; Dalian, Liaoning, China). The amplification conditions were as follows: template denaturation and polymerase activation at 95 °C for 30 s, followed by 40 cycles of amplification at 95 °C for 5 s, 60 °C annealing for 30 s and 72 °C extension for 30 s. 30 embryos were used in each assay, and the data are representative of three independent assays, and the levels of mRNAs were calculated using the $2^{-\Delta\Delta CT}$ method. The specific primers

for each gene were designed as follows: GAPDH, forward 5'-AATGGATTTGGACGCATTGGT-3' and reverse 5'-TTTGCACTGGTACGTGTTGAT-3';

IGF-1, forward 5'-GGTGGATGCTCTTCAGTTCGTG-3' and reverse 5'-TCCACAATGCCTGTCTGAGGTG-3';

IGF-1R, forward 5'-TGTCCAGCCGAAGCAGGAACA-3' and reverse 5'-TCTGCTGGCATGGTTCTCGC-3';

IGF-2 forward 5'-TCAGAGAGGCCAAACGTCATCG-3' and reverse 5'-TGGTTGCTGGACATCTCCGAAG-3';

IGF-2R forward 5'-CTCCTTGGACAAGCAGACGTGC-3' and reverse 5'-ATGCTTCGTAGCCACCAGTGCG-3';

EGF forward 5'-TCAATGGTGGCGTGTGCATG-3' and reverse 5'-CAGCCACCACCATGATGTCATG-3';

EGFR forward 5'-ACTGCTGCCACAACCAATGTGC-3' and reverse 5'-GCATGTGGCCTCATCTTGGAAC-3';

TGF-α forward 5'-CCTGGTGGTGGTCTCCATTGTG-3' and reverse 5'-TCAGAGTGGCAGCAAGCAGTCC-3'.

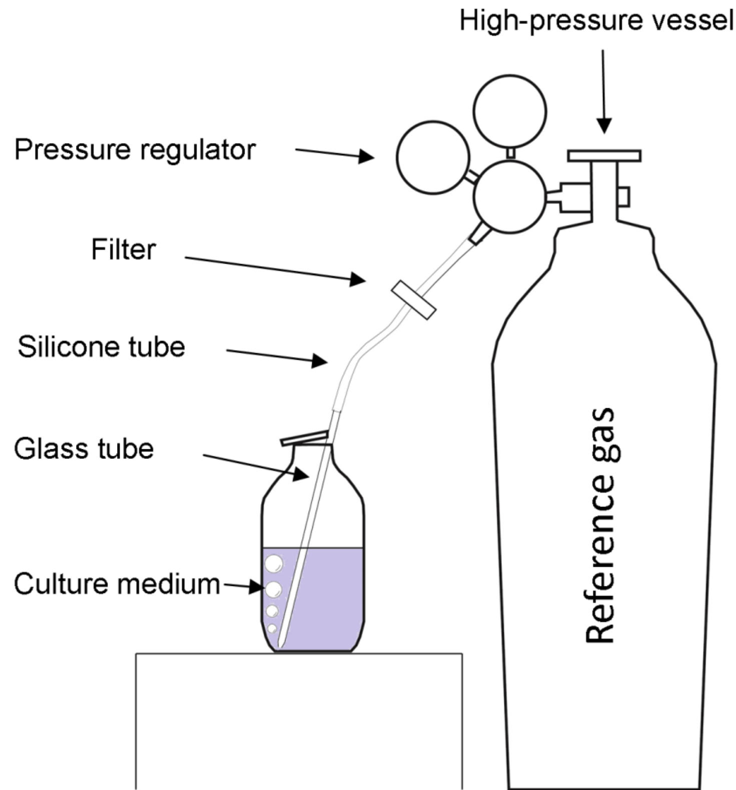

Figure S1. Schematic of how the culture medium was aerated with reference gas.

The process of aeration is similar to supplying addition oxygen to a fishbowl. Briefly, 100 mL culture medium was put into a 200 mL glass bottle, then the medium was injected with reference gas by a glass tube which connected to the pressure vessel with two silicone tubes and a filter. The diameter of one end of the glass tube is about 1 mm. Gas was in jected into the medium at the rate of 2 to 3 bubbles per second. The culture medium were aerated for 2 h at room temperature before sealed-culture, after aeration, 200 μL medium and embryos were quickly loaded into 200 μL PCR tubes, then the PCR tubes were sealed immediately with melted wax and cultured at 37° C.

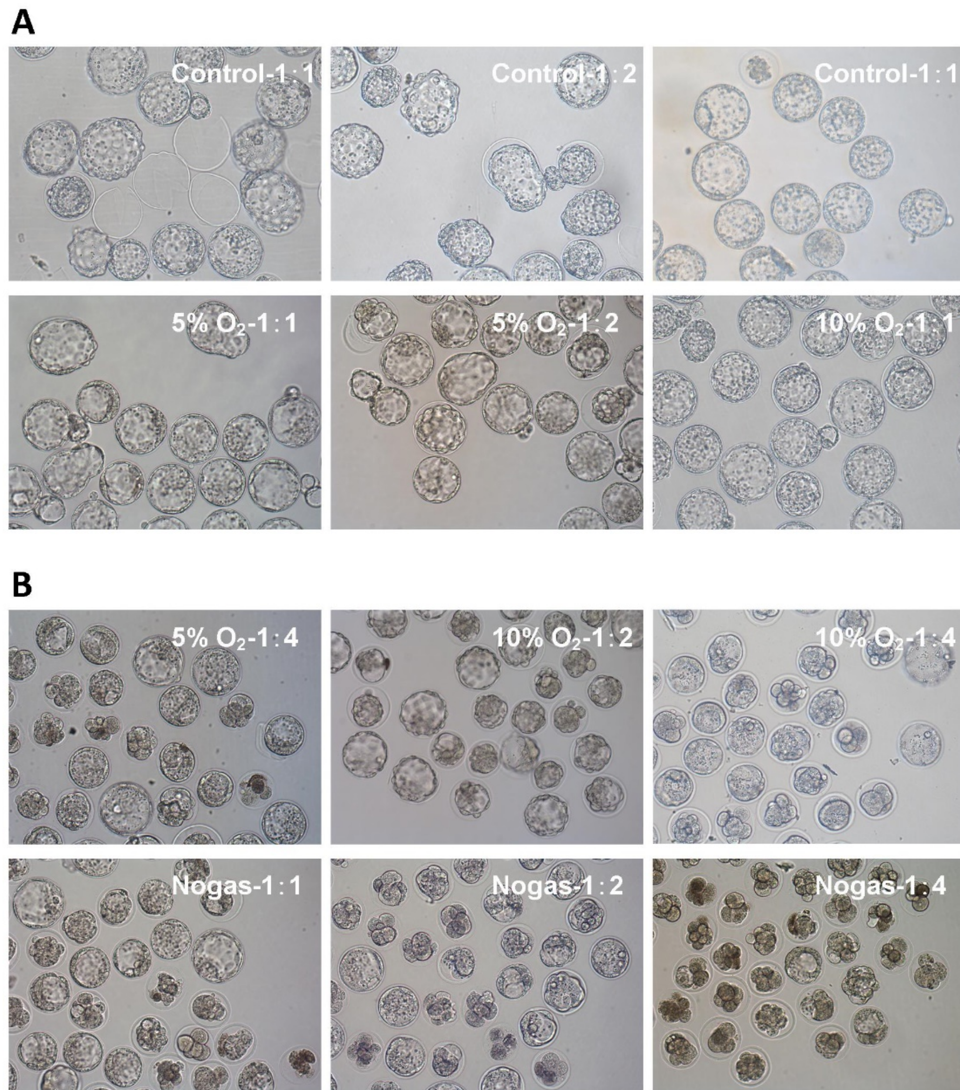

Figure S2. The morphology of embryos cultured in different groups for 60 h. Embryos in control groups and sealed-culture groups with healthy morphology (A); Embryos in sealed-culture groups with poor morphology (B). Magnification 200×.

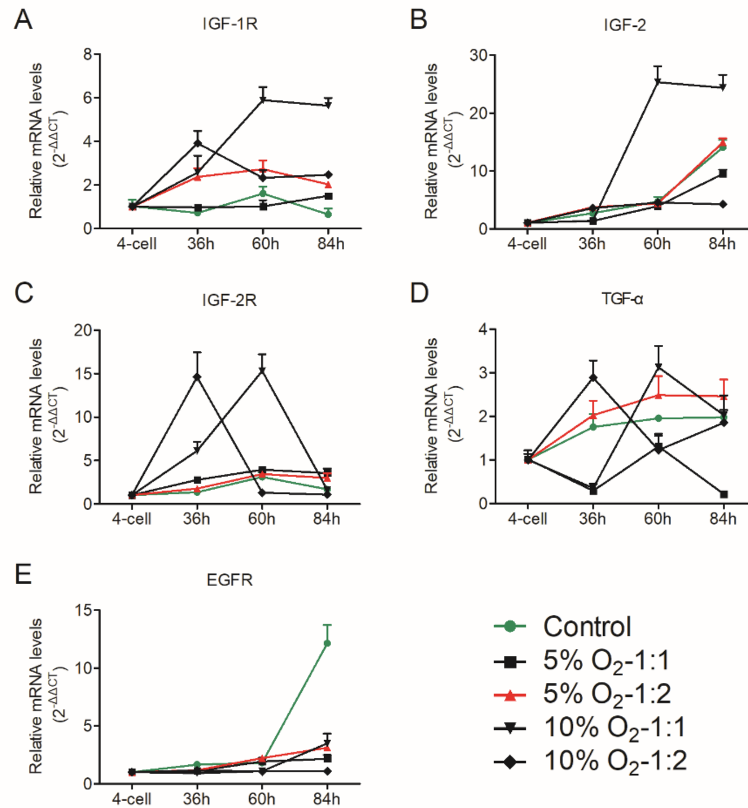

Figure S3. The expression patterns of growth factors under different culture conditions.

The 4-cell embryos in different culture conditions were cultured for 36 h, 60 h and 84 h, respectively. The expression patterns of IGF-1, IGF-1R, IGF-2, IGF-2R, EGFR, TGF-α and EGFR were analyzed by RT-qPCR. A-E show similar expression patterns of IGF-1R, IGF-2, IGF-2R, TGF-α and EGFR in 5% O_2-1:2 sealed-culture group and the control group, however, IGF-1 and EGF were detected in none of the groups in any embryo stage. Independent experiments were performed three times, and the data are expressed as $2^{-\Delta\Delta CT}$.

Table S1. The rates of embryo stopped in different stages after 84 h sealed-culture

4-cell stage embryos were developed in different sealed-culture systems and general control conditions for 84 h, and then the stages of embryos were analyzed. % 4-cell represented the rates of embryos stopped at 4-cell stage; % 8-cell represented the rates of embryos stopped at 8-cell stage; % Moru represented the rates of embryo stopped at morula stage; % Blas represented the rates of embryos developed to blastocyst stage but not hatching at that moment; % Hatch represented the hatching rates of embryos; % Frag represented the rates of fragment embryos which could not be recognized the developmental stage. Data are the mean ± S.D. Within columns, values with different superscript letters indicates $p<0.05$. Independent experiments were performed three times for each group.

Groups	% 4-cell	% 8-cell	% Moru	% Blas	% Hatch	% Frag
5% O_2 1:1	$1.6±1.0^{abc}$	$1.2±1.1^{b}$	$6.0±3.4^{bc}$	$59.6±1.7^{a}$	$30.7±4.7^{b}$	$1.0±1.7^{c}$
5% O_2 1:2	$0.7±0.6^{bc}$	$0.3±0.6^{b}$	$4.7±3.8^{bc}$	$30.8±3.7^{bc}$	$62.2±8.9^{a}$	$1.3±1.2^{c}$
5% O_2 1:4	$6.2±2.0^{ab}$	$4.3±4.6^{b}$	$48.6±8.4^{a}$	$28.4±9.2^{bc}$	$6.2±3.8^{c}$	$6.2±6.6^{bc}$
10% O_2 1:1	$0.8±1.0^{abc}$	$0.7±0.6^{b}$	$6.2±2.7^{bc}$	$32.6±3.1^{b}$	$58.9±6.9^{a}$	$0.8±1.4^{c}$
10% O_2 1:2	$4.7±2.5^{abc}$	$9.6±2.1^{ab}$	$6.0±2.6^{bc}$	$32.2±10.3^{b}$	$30.6±2.5^{b}$	$16.9±5.9^{b}$
10% O_2 1:4	$6.8±3.3^{ab}$	$21.9±15.0^{a}$	$27.3±25.3^{ab}$	$6.2±7.5^{d}$	$1.4±2.4^{c}$	$36.4±13.0^{a}$
No gas 1:1	$2.2±1.9^{abc}$	$3.2±0.3^{b}$	$8.7±2.5^{bc}$	$74.0±3.5^{a}$	$6.2±4.8^{c}$	$5.7±3.1^{bc}$
No gas 1:2	$3.8±2.5^{abc}$	$7.2±2.3^{b}$	$8.2±3.1^{bc}$	$60.5±5.2^{a}$	$16.0±2.9^{bc}$	$4.3±2.9^{bc}$
No gas 1:4	$6.9±3.4^{a}$	$10.3±1.9^{ab}$	$28.4±8.7^{ab}$	$14.5±1.8^{cd}$	$6.9±1.1^{c}$	$33.0±6.9^{a}$
Control 1:1	$0.0±0.0^{c}$	$0.3±0.6^{b}$	$4.3±1.2^{bc}$	$26.0±4.6^{bc}$	$69.0±5.6^{a}$	$0.3±0.6^{c}$
Control 1:2	$0.7±1.2^{bc}$	$0.6±1.1^{b}$	$1.3±1.1^{c}$	$42.1±3.5^{b}$	$55.3±4.2^{a}$	$0.0±0.0^{c}$
Control 1:4	$1.3±2.3^{abc}$	$0.0±0.0^{b}$	$4.0±4.0^{bc}$	$37.3±6.1^{b}$	$56.0±6.9^{a}$	$1.3±2.3^{c}$