Coculture with a Human Granulosa Cell Line Enhanced the Development of Murine Preimplantation Embryos Via SCF/c-Kit System

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Purpose: To evaluate the effect on the coculture of murine embryos with a human ovarian granulosa tumor derived cell line (KGN cells).

Methods: We observed microscopically the growth of murine preimplantation embryos in the coculture system with KGN cells or in the presence with exogenous stem cell factor (SCF). The reverse transcriptase–polymerase chain reaction (RT-PCR) method was used to analyze the gene expression of SCF in KGN cells cocultured with murine embryos.

Results: The coculture system with KGN cells significantly increased the rate of embryo development to late blastocyst and to hatching stage. We also found that coculture with murine embryos enhanced the gene expression of SCF in KGN cells. Adding human recombinant SCF to the medium significantly enhanced embryo development to late blastocyst and hatching stage.

Conclusions: KGN cells may facilitate preimplantation embryo development through SCF/c-kit paracrine system.

KEY WORDS: Coculture; embryo development; KGN cells, SCF/c-kit;

INTRODUCTION

The low pregnancy rate in human in vitro fertilization–embryo transfer (IVF-ET) programs is partially caused by suboptimal conditions in embryo culture. Embryo development might be modified by the presence of supportive surrounding cells in vivo. For example, a study showed that coculture with granulosa cells enhances human embryo development, implantation, and pregnancy rate for IVF-ET (1). The oviduct and endometrium may either secrete embryotrophic factors (growth factors, cytokines, and other proteins) (2) or stabilize the culture medium by detoxifying and removing

undesirable factors from the medium. Mutual interaction between embryos and somatic cells may play an important role in preimplantation embryonic development.

Murine preimplantation embryos have been shown to express receptors for various growth factors. These include the receptors for stem cell factor (SCF), leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), and epidermal growth factor (EGF) (3–6). All these factors may mediate the positive effects for embryo development as paracrine factors (6–10). In the present study, we evaluated the effect of a coculture system with murine preimplantation embryos and human ovarian granulosa tumor derived cells (KGN cells) (11). We further examined whether and how SCF, LIF, and bFGF interacts between murine embryos and KGN cells via paracrine manner.

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MATERIAL AND METHODS

Collection of Murine One-Cell Embryos

Ovulation was stimulated in fifty B6C3F1 female mice aged 7 weeks (Shimizu Experimental Animals Laboratory, Kyoto, Japan) by an i.p. injection of 5 IU of pregnant mare serum gonadotropin (gonadotropin; Sigma, St. Louis, MO), followed 48 h later by an injection of 5 IU of human chorionic gonadotropin (HCG; Mochida co., Tokyo, Japan). Immediately thereafter, the mice were mated overnight with males of the same strain. After 18 h, we collected one-cell embryos from the resected oviducts, using modified human tubal fluid medium (HTF; Irvine Scientific, Irvine, CA). Next, we pipetted and removed carefully the cumulus granulosa cells by hyaluronidase (1 mg/mL, Sigma). We removed the murine granulosa cells to examine precisely the function of feeder cells in the experiment of coculture system. All experiments were performed in accordance with the guidelines of the Tottori University Committee for Animal Experimentation.

Reagent

Recombinant human SCF, LIF, bFGF, and anti-human SCF antibody were purchased from Genzyme Techné (Minneapolis, MN), Pepro Tech, Inc. (Rocky Hill, NJ), and Collaborative Biomedical Products (Bedford, MA), respectively. Rat anti-mouse monoclonal antibody to the extracellular domain of the c-kit receptor, ACK2 was provided by Dr H. Yamasaki (Tottori University Faculty of Medicine, Yonago, Japan). Detailed characteristics of ACK2 have been described previously (12).

Embryo Culture

The embryos were cultured with a mini-incubator (MINC-1000, COOK IVF, Brisbane, Australia; 6% CO₂/5% O₂/89% N₂, mixed gas) at 37°C. We used the HTF medium with 10% SSS (synthetic serum substitute, Irvine Scientific), which is commonly utilized for human IVF-ET program. The developmental stage and morphology of each embryo was assessed and recorded for 96 h. After 96 h of incubation, blastocysts were graded on the basis of the expansion of the blastocoele cavity as follows: early blastocyst—compacted embryos with the beginnings of a blastocoele cavity or with a cavity less than half of the embryo volume; blastocyst—embryos with a blastocoele cavity occupying half to two thirds of the embryo

volume; late blastocyst—embryos with a blastocoele cavity occupying the entire volume of the embryo in which initiated herniation of the zona pellucida by the trophectoderm may have begun.

Coculture System

Nishi *et al.* established KGN, a steroidogenic human ovarian granulosa like tumor cell line, from a patient with invasive ovarian granulosa cell tumor. In their study, KGN cells secreted pregnenolone and progesterone, both of which increased after stimulation with (Bu)₂ cAMP. However, they observed little or no secretion of 17 beta-hydroxylated steroids, dehydroepiandrosterone, androstenedione, or estradiol (11). KGN cells were plated at a density of 30,000 cells per well (4-well multidish; Nunc A/S, Roskilde, Denmark) on the day before the onset of coculture at 37°C under an atmosphere of 5% CO₂/95% air, in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (DMEM/FBS). Five murine one-cell embryos per well were transferred with the monolayer of KGN cells and then cultured in 1 mL of the HTF medium with 10% SSS with a mini-incubator for 96 h.

RT-PCR and Southern Blot Analysis

KGN cells were grown to subconfluent in 35-mm tissue culture dishes (Nunc A/S) for 24 h, then one-cell murine embryos from the oviducts (about 40 embryos) were cocultured at a ratio of 1 embryo to 5000 KGN cells (13). After 96 h, total RNA was extracted from KGN cells and murine embryos by the Rneasy Mini Kit (Qiagen Inc., Valencia, CA). The reverse transcription of RNA from these cells into complementary DNA and PCR amplification was performed using a Gene Amp RNA and PCR Core Kit (Perkin Elmer, Branchburg, NJ). RNAs (1 µg) extracted from each sample were used. Each sample was amplified for 35 cycles. The cycles consisted of 30 s at 94°C, 30 s at 58°C, 90 s at 72°C. For the RT-PCR, the specific primers for SCF, LIF, bFGF, and the G3PDH were used (14–16). We confirmed the authenticity of the PCR products obtained from the experiments by Southern blot analysis. These procedures were previously described in detail (6). The details of the primers, internal probes, and PCR products are shown in Table I. Densitometric analysis of the PCR products was performed using a public domain National Institutes of Health (NIH) image program (written by Wayne Rasband, NIH). The densitometric

Table I. Primer Pairs and Probes Used for RT-PCR and Southern Blotting

Gene	Primer	Primer sequence	Amplified length (bp)
SCF	sense	5'CCAGAACCCAGGCTCTTTAC3'	316
	antisense	5'AGGCTCCAAAAGCAAAGC3'	233
	probe	5'AGCCATGGCATTGCCAGC3'	
LIF	sense	5'CTGTTGGTTCTGCACTGGA3'	348
	antisense	5'GGGTTGAGGATCTTCTGGT3'	
	probe	5'TCACAACAACCTCATGAACC3'	
bFGF	sense	5' TCAAAAGTTCGGCATGTA 3'	250
	antisense	5'TGGGGAAGAAATATCCATC3'	
	probe	5'TTTTGATCAGTTAACTTGA3'	
G3PDH	sense	5'ACCACAGTCCATGCCATCAC3'	450
	antisense	5'TCCACCACCCTGTTGCTGTA3'	

values of SCF, LIF, and bFGF mRNAs were normalized with the corresponding G3PDH mRNAs.

Statistical Analysis

All experiments were repeated at least three times. The analysis of variance (ANOVA) and χ^2 test were used to analyze the rates of embryo development. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Coculture with KGN Cells on Murine Embryos Development

We found that the rate of development to late blastocyst (78.4% vs. 65.5%) and to hatched blastocysts (48.1% vs. 30.4%) were enhanced significantly in the presence of KGN cells compared with absence of the cells (Table II).

Effects of Exogenous SCF, LIF, and bFGF on Murine Embryos Development

Adding recombinant SCF (50 ng/mL) or LIF (50 ng/mL) to HTF medium significantly increased the rate of development to late blastocyst (82.2% or 85.2% vs. 65.5%) and to hatching stage (62.2% or

67.6% vs. 30.4%), respectively (Table III). In contrast, bFGF did not affect embryo development. Adding anti-SCF antibody (5 μ g/mL) and ACK2 (5 μ g/mL, antibody to the c-kit) to the coculture media with KGN cells apparently reduced the development rate. Anti-SCF antibody or ACK2 alone had no detectable effects on the embryo development (Table IV).

Gene Expressions of SCF, LIF, and bFGF in KGN Cells Cocultured with Murine Embryos

RT-PCR was performed to analyze mRNA expression of SCF, LIF, and bFGF in the KGN cells. We observed the SCF mRNAs of the long form of 316-bp band corresponding to the secreted form that include exon 6, and the short form of 233-bp band corresponding to the membrane bound form that lack exon 6. Interestingly, the expression of SCF mRNA in KGN cells was remarkably increased by the coculture with murine embryos. Like SCF, the expression of LIF mRNA was enhanced in the KGN cells cocultured with murine embryos (Fig. 1).

DISCUSSION

Although advances in assisted reproductive technology have been rapid in recent years, the pregnancy rate of embryos in human IVF programs remains

Table II. Effects of the Presence of KGN Cells on the Development of Murine Embryos After 96 h of Culture

KGN coculture	No. of oocytes	No. and % of embryos developed to			
		Early blastocyst	Blastocyst	Late blastocyst	Hatched blastocyst
+	102	95 (93.1%)	89 (87.2%)	80 (78.4%)*	52 (48.1%)**
—	148	130 (87.8%)	115 (77.0%)	97 (65.5%)*	45 (30.4%)**

* $P < 0.05$; ** $P < 0.01$.

Table III. Effects of Exogenous SCF, LIF, and bFGF on the Development of Murine Embryos After 96 h of Culture

Treatment	No. of oocytes	No. and % of embryos developed to			
		Early blastocyst	Blastocyst	Late blastocyst	Hatched blastocyst
Control	148	130 (87.8%)	115 (77.0%)	97 (65.5%)*	45 (30.4%)**
SCF (50 ng/ML)	45	42 (93.3%)	39 (86.6%)	37 (82.2%)*	28 (62.2%)**
LIF (50 ng/ML)	34	32 (94.1%)	30 (88.2%)	29 (85.2%)*	23 (67.6%)**
bFGF (50 ng/ML)	36	33 (91.6%)	28 (77.8%)	24 (66.7%)	14 (38.8%)

* $P < 0.05$; ** $P < 0.01$.

unsatisfactory. The specific requirements for in vitro culture for early stage embryos are still not fully understood. Under in vivo conditions, the embryos and cumulus granulosa cells are in an optimal condition to achieve successful fertilization and progress through early embryogenesis to implantation.

The beneficial effects of feeder cells on embryo development may not be dependent on cell type or tissue origin. Murine embryos showed equally enhanced developmental rates and reduced fragmentation when cocultured with human granulosa lutein cells, cumulus granulosa cells, and epithelial or fibroblast cells from oviduct and endometrium (17). The coculture systems of human embryos with different cell types, such as human ampullary cells (18), human granulosa cells (19), Vero cells (derived from green monkey kidneys) (20), bovine oviductal epithelial cells (21), and human ovarian cancer cells (22) were found to be successful in improving embryo development in vitro. Results from this study demonstrated similar beneficial effects on embryo development when murine embryos were cocultured with KGN cells. The coculture system has great potential for future application in human-assisted reproductive technology. These include enhancing the development of embryos to the blastocyst stage for blastocyst transfer, blastocyst biopsy for genetic diagnosis, blastocyst cryopreservation, and assisted hatching. Although we stated the advantage

of coculture system, it is necessary to consider the safety and propriety of using both nonautologous and tumor-derived cell lines with human embryos. A serious concern regarding the clinical use is the potential for the transmission of infectious disease. Experience with organ allotransplantation has shown that diseases such as human immunodeficiency virus (HIV) infection, Creutzfeldt-Jakob disease, and hepatitis B or C virus infection can be transmitted. If we resolve this problem, the coculture system with nonautologous cells will be utilized for human IVF program.

KGN cells are undifferentiated and maintain the physiological characteristics of granulosa cells in the follicular phase, including the same pattern of steroidogenesis and Fas-mediated apoptosis as those observed in normal granulosa cells (11). Although some investigators have established ovarian granulosa cell lines, KGN is the only cell line that expresses functional FSH receptor. The feeder cells, like KGN, can impart a beneficial effect on embryo development, via two main mechanisms: first, the increased metabolic rate of the proliferative feeder cells consumes much of the existing oxygen in the medium. Furthermore the feeder cells may stabilize the culture medium by detoxifying and reducing undesirable factors, such as oxidative free radicals. Second, the secretion of embryotrophic growth factors by the feeder

Table IV. Effects of Anti-SCF Antibody or ACK2 and the Presence of KGN Cells on the Development of Murine Embryos After 96 h of Culture

Treatment			No. of oocytes	No. and % of embryos developed to			
KGN colculture	Anti SCF Ab	ACK2		Early blastocyst	Blastocyst	Late blastocyst	Hatched blastocyst
+	—	+	48	21 (43.8%)**	18 (37.5%)**	12 (25.0%)**	3 (6.3%)**
—	—	+	39	33 (84.6%)**	30 (76.9%)**	24 (61.5%)**	13 (33.3%)**
+	+	—	28	15 (53.6%)*	14 (50.0%)*	8 (28.6%)**	2 (7.1%)**
—	+	—	30	25 (83.3%)*	24 (80.0%)*	19 (63.3%)**	9 (30.0%)**
+	—	—	102	95 (93.1%)	89 (87.2%)	80 (78.4%)	52 (48.1%)
—	—	—	148	130 (87.8%)	115 (77.5%)	97 (65.5%)	45 (30.4%)

* $P < 0.05$; ** $P < 0.01$.

Fig. 1. Expression of SCF, LIF, and bFGF mRNAs in the KGN cells. The PCR products for SCF (316 and 233 bp), LIF (348 bp), and bFGF (250 bp), and internal control G3PDH (450 bp) are indicated. Lane 1, KGN cells; Lane 2, KGN cells cocultured with murine embryos. Specificity of each PCR product was confirmed by Southern blot analysis.

cells into the culture media may foster embryonic development.

Interestingly, the results of this study suggest that SCF and LIF gene expression of KGN cell are apparently stimulated by coculture with murine embryos, suggesting that murine embryos may give some signals that activate the transcripts of SCF or LIF genes in KGN cells. In addition, we proved that adding exogenous SCF significantly increased the rate of embryo development to late blastocyst and hatching stage, and that adding anti-SCF antibody and ACK2 to the coculture media with KGN cells reversed the beneficial effects of coculture. Therefore, SCF/c-kit system may be involved as one of paracrine signals in development of murine preimplantation embryos in coculture system.

SCF transcript was shown to be expressed in human or murine granulosa cells (3,14). In mice, the expression of c-kit mRNA was observed from the late two-cell stage to the expanded and hatched blastocyst (3). We previously demonstrated that SCF significantly promoted the expansion of the surface area of the spreading blastocysts (7). On the other hand, LIF and LIF receptor (LIF-R) transcripts were detectable in human preimplantation embryos (4). It

was reported that embryos cultured on Vero cells that express LIF reached the blastocyst stage at a high rate (23). In addition, blastocyst-hatching rates improved in the presence of recombinant human LIF (24). Thus, SCF/c-kit or LIF/LIF-R may have an important role on the interaction between embryos and granulosa cells as paracrine factors.

In conclusion, we revealed the beneficial effects of a coculture system with KGN cells on the development of murine embryos. The stimulatory effects of SCF suggested that SCF/c-kit system works in the local environment of the embryos. Further studies are necessary to characterize the coculture effect in order to provide specific components to the medium or to remove toxic substances, and thereby offer infertile couples a safe and successful embryo culture system. The establishment of a culture environment makes it possible to elucidate the metabolic and nutritional requirements of embryos and improve the efficient development of embryos for practical use.

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