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Ghrelin Inhibits the Development of Mouse Preimplantation Embryos *in Vitro*

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Although ghrelin acts as a modulator of feeding behavior and energy metabolism in the central nervous system, recent studies have implicated the peripheral actions of ghrelin in reproductive tissues. Here, we investigated the expression of ghrelin and its receptor (GHS-R) in mouse oocyte and preimplantation embryos, and we examined the role of ghrelin in the regulation of early embryo development. Both ghrelin and GHS-R mRNAs were detected in morula or more advanced embryo stages. As for the origin of ghrelin, both ghrelin mRNA and protein were identified in the uterine endometrium. The levels of ghrelin in uterine fluid as well as plasma were significantly increased in fasting mice compared with animals with free access to foods. Addition of ghrelin to culture media inhibited the development of two-cell embryos to the hatched blastocysts, and the inhibitory effects of ghrelin were abolished by an antagonist for the GHS-R. In addition, ghrelin significantly decreased the number of total cells, inner cell mass, and trophectoderm cells in blastocysts. These observations suggest that ghrelin could inhibit the development of preimplantation embryos during fasting. Thus, ghrelin may act as a peripheral factor to avoid the excess metabolic demands imposed by pregnancy during malnutritional states. (*Endocrinology* 144: 2623–2633, 2003)

GH SECRETAGOGUES (GHS) are a group of artificially synthesized peptidyl and nonpeptidyl molecules known to release GH *in vivo* by acting through a seven-transmembrane, G protein-coupled receptor, GHS-receptor (GHS-R; Refs. 1–6). Recently, ghrelin was identified as an endogenous ligand for the GHS-R (6). Ghrelin is a 28-amino acid peptide with an essential n-octanoyl modification at the Ser^3 residue and is primarily expressed in neuroendocrine X/A-like cells of the gastric mucosa, pituitary gland, and hypothalamus (7, 8). The structure of ghrelin is highly conserved between rodents and human with changes in only two residues (6). In addition to its potent GH-releasing activity, ghrelin stimulates food intake through the modulation of the expression of hypothalamic neuropeptide Y (NPY) and/or the agouti-related protein (9–11). Ghrelin induces adiposity in rodent by increasing food intake and reducing fat utilization (12). Thus, ghrelin is considered to play an important role in the regulation of feeding behavior and energy metabolism by mainly acting at the central nervous system (9–12).

Although ghrelin is expressed in the central nervous system to regulate diverse functions, ghrelin transcripts have also been detected in several peripheral tissues, such as kidney, hematopoietic immune cells, placenta, lung, pancreas, testis, and stomach (13–19). In addition, the GHS-R is expressed in diverse peripheral tissues (14, 20). Although the exact functional significance of peripheral ghrelin is unknown, ghrelin inhibits the secretion of testosterone by testis Leydig cells under the stimulation of human chorionic gonadotropin (hCG) and cAMP (19), thus suggesting novel roles of ghrelin in the reproductive system.

In animals with insufficient nutrient intake, the fertility potential is suppressed, probably due to adaptive responses to evade the excess metabolic demands imposed by pregnancy (21, 22). The level of plasma ghrelin was known to elevate under malnutritional states (12, 23–26). Some GHS, including ghrelin, were shown to negatively regulate cell viability and proliferation (27–29). Ghrelin was demonstrated to inhibit the proliferation of breast cancer cells (29). These findings have provided the basis to hypothesize that ghrelin may inhibit development of embryos when maternal nutrient intake is insufficient.

The aim of this study was to investigate whether ghrelin could inhibit development of preimplantation embryos in mice. We sought to determine 1) the temporal expression of ghrelin and GHS-R mRNAs in oocytes and preimplantation embryos up to the hatched blastocyst stage; 2) whether ghrelin is secreted by the reproductive tracts and binds to preimplantation embryos; and 3) the effects of ghrelin treatment on preimplantation embryo development. Our results demonstrate that mouse preimplantation embryos express both ghrelin and GHS-R, and ghrelin secreted from reproductive tracts could inhibit the development of early embryos through the GHS-R.

Materials and Methods

Collection of mouse oocytes and preimplantation embryos

Female IVCS mice, aged 9 wk, (Institute for Animal Reproduction, Ibaragi, Japan) were superovulated with a single ip injection of 10 IU of

Abbreviations: EIA, Enzyme immunoassay; FAM, Tri-5 (and Tri-6) carboxyfluorescein; GHRP-6, GH-releasing peptide-6; GHS, GH secretagogue(s); GHS-R, GHS receptor; hCG, human chorionic gonadotropin; HTF, human tubal fluid; ICM, inner cell mass; NPY, neuropeptide Y; TE, trophectoderm.

pregnant mare serum gonadotropin (Sigma, St. Louis, MO), followed 48 h later by 10 IU of hCG (Sigma). Two-cell stage embryos were obtained by flushing the oviducts of the mated mice at 46–47 h after hCG injection. The embryos were washed three times with M2 medium (Sigma). Subsequently, groups of 10–15 embryos were placed in 30-μl drops of the human tubal fluid (HTF) medium (30), covered by mineral oil, and cultured at 37 C in 5% CO_2 in air. For RT-PCR analysis, four-cell, eight-cell, morula, blastocyst, and hatched blastocyst-stage embryos were collected from cultures in individual micro-drop at 50–52, 68–70, 90–92, 118–120, and 142–144 h after hCG injection, respectively. Unfertilized oocytes were also obtained from oviducts of unplugged mice at 18–20 h after hCG injection.

All procedures involving the care and use of animals were approved by the Animal Research Committee, Akita University School of Medicine (Akita, Japan).

RT-PCR and nested PCR

The methods of RT-PCR for oocytes and preimplantation embryos were described previously (31, 32). Briefly, poly $(A)^+$ mRNA was isolated from 15 mouse oocytes or preimplantation embryos of several stages (two-cell, four-cell, eight-cell, morula, blastocyst, and hatched blastocyst), and each mRNA sample was reverse transcribed into cDNA. Exogenous rabbit α-globin mRNA (Life Technologies, Inc., Rockville, MD) was added to each sample before mRNA extraction to evaluate the efficiency of mRNA extraction and the RT procedure. The amount of cDNA subjected to each PCR was equivalent to the number of genomes (*e.g.* one two-cell stage embryo or one quarter of an eight-cell stage embryo), so that each PCR product was derived from the same number of transcribing genomes.

The primers for ghrelin and GHS-R were based on GenBank accession no. AB035701 and AF332997, respectively, as shown in Table 1. The PCR was performed according to the programs described in the legend of Table 1. For positive controls for ghrelin and GHS-R, mouse placenta and brain cDNAs were also amplified. For negative controls, the specimen in which water was substituted for mRNA was amplified. Because of the low number of oocytes and preimplantation embryos under study, heminested PCR was needed to obtain optimal results. Furthermore, total RNA was extracted from uterus of pregnant mice at d 4.5 after mating, and RT-PCR for ghrelin was performed.

The PCR products were separated by 2% agarose gel electrophoresis (Agarose-LE, Nacalai Tesque, Inc., Kyoto, Japan) in the presence of ethidium bromide (Sigma), and visualized with a UV transilluminator (Funakoshi, Tokyo, Japan). To confirm identity, bands of each PCR product were eluted from the agarose gel using the QIAquick gel extraction kit (QIAGEN KK, Tokyo, Japan), ligated into the pDrive Cloning vector (QIAGEN KK), and cloned in accordance with standard protocols. Plasmid DNA was recovered using Quantum Prep Plasmid Miniprep kit (Bio-Rad Laboratories, Inc., Hercules, CA), cycle sequenced, and analyzed in a ABI 100 DNA sequencer (PE Applied Biosystems, Tokyo, Japan) using T7 or SP6 site-specific primers.

Binding of fluorescent ghrelin to mouse preimplantation embryos

For binding studies, two-cell stage embryos were cultured in the HTF medium at 37 C in 5% CO_2 in air. When the embryos reached the four-cell or blastocyst stage, the medium was replaced with 1, 10, and 100 nM of fluorescent ghrelin conjugated with Tri-5 (and Tri-6) carboxyfluorescein (FAM; Phoenix Pharmaceuticals, Inc., Belmont, CA) in the HTF medium and incubated for 30 min at 37 C. After three washes, these embryos were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed three times. To determine the background fluorescence, embryos were incubated only with the HTF medium. Nonspecific binding of FAM-ghrelin (100 nM) was estimated by coincubating with a 100-fold excess of unlabeled ghrelin (Phoenix Pharmaceuticals, Inc.). The fluorescence signals in embryos were visualized using a confocal laser scanning microscope (LSM 410, Carl Zeiss, Oberkochen, Germany). Optical sections with intervals of 1 μm were taken with a 63×/1.4 Plan Apochromat objective, and the fluorescent images were obtained as 8-bit images of TIF format files. After the calculation of total numbers of pixels for the whole embryo, the numbers of fluorescent pixels, which correspond to the binding site of FAM-ghrelin to GHS-R, were measured by SigmaScan Pro 5.0 (SPSS Japan Inc., Tokyo, Japan).

Immunohistochemistry

Uteri were obtained from 9-wk-old pregnant mice at d 4.5 after mating and fixed with 4% paraformaldehyde in PBS for 6 h at 4 C. Fixed frozen tissue sections were blocked with 10% normal goat serum (DAKO Corp., Kyoto, Japan) for 30 min at room temperature. Samples were incubated with rabbit antighrelin serum (Phoenix Pharmaceuticals, Inc.) with a dilution of 1:150 in 1% PBS-BSA/0.1% Triton X-100 (Sigma), overnight at 4 C. After three washes in cold PBS, samples were incubated with 1.0 μg/ml of goat antirabbit Cy3 fluorescein antibody (Chemicon, Temecula, CA) in 1% PBS-BSA/0.1% Triton X-100, for 1 h at room temperature in the dark. After three washes in cold PBS, slides were covered in a drop of antifade mounting medium (DAKO Corp.) and analyzed under an epifluorescence microscope (Olympus Corp., Tokyo, Japan). For negative controls, sections were subjected to the same method, except that the primary antiserum was replaced by the same dilutions of normal rabbit serum (DAKO Corp.) or by the primary antiserum preabsorbed with the ghrelin peptide (Phoenix Pharmaceuticals, Inc.) at 50 μg/ml.

Enzyme immunoassay (EIA)

Ghrelin concentrations in plasma and uterine fluid were measured using a ghrelin EIA kit (Phoenix Pharmaceuticals, Inc.) with a sensitivity of 0.9 ng/ml, and the intra- and interassay coefficients of variation were less than 5% and 14%, respectively. For measurement of ghrelin in the uterine fluid, uteri were collected from 10 nonpregnant 9-wk-old mice at the day of estrus and 9-wk-old pregnant mice at d 4.5 after mating, all with free access to a standard rodent diet. This pregnant stage corresponded to the embryonic stage of blastocyst. After 48 h of fasting (only access to water was allowed), samples were also obtained from 10

TABLE 1. Primers used for RT-PCR and nested PCR, PCR cycles and temperatures for amplification of the different cDNA

Transcript	PCR round		Primer sequence (5′-3′)	Size of product (bp)	Annealing (C)
Ghrelin	1st	Sense	CCATCTGCAGTTTGCTGCTA	394	60
		Antisense	CGGATGTGAGTTCTTGCTCA		
	2nd	Sense	CCATCTGCAGTTTGCTGCTA	350	60
		Antisense	GCCTGTCCGTGGTTACTTGT		
GHSR1a	1st	Sense	CTGCTCTGCAAACTCTTCCA	375	64
		Antisense	CTTCCTCCCGATGAGACTGT		
	2nd	Sense	CTGCTCTGCAAACTCTTCCA	354	64
		Antisense	GAGCACAGTGAGGCAGAAGA		
β-actin		Sense	GGACCTCACTGACTACCTCATGAA	524	55
		Antisense	GGTGGAAGGTGGTCAACACCTAG		
α-globin		Sense	GCAGCCACGGTGGCGAGTAT	257	55
		Antisense	GTGGGACAGGAGCTTGAAAT		

PCR cycles: denaturation at 94 C for 30 sec and extension at 72 C for 30 sec, total 35 cycles were performed.

nonpregnant mice at the day of estrus and at d 4.5 after mating. Uterine fluid samples were obtained by flushing the uterine cavities with 10 μl of 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate buffer (Sigma) containing 0.6 TIU/ml of aprotinin (Sigma) and centrifuged at 2000 × *g* for 5 min. Each supernatant was stored at −70 C until assay. The plasma samples were obtained from the same mice described above and stored at −70 C until assay. Ghrelin content in uterine fluid and plasma samples was measured by EIA in duplicate.

Embryo cultures

Two-cell stage embryos were collected as described above. The embryos were washed three times with the M2 medium (Sigma). Groups of 10–15 embryos randomly selected were placed in 30-μl drops of HTF medium covered by mineral oil with or without synthetic rat ghrelin (Phoenix Pharmaceuticals, Inc.). The mature peptide of rat ghrelin is identical to the mouse ghrelin (Ref. 6; GenBank accession no. AB035701). Embryos were cultured over 72 h up to the hatched blastocyst stage at 37 C in 5% CO_2 in air.

To examine whether the effects of ghrelin on preimplantation embryos were mediated through GHS-R, embryos were cultured in HTF medium containing 10 mM of an antagonist for GHS-R, [D-Lys-3]GH-releasing peptide-6 (GHRP-6) (Peninsula Laboratories, Inc., San Carlos, CA; Ref. 10) with or without 100 nM of ghrelin. Furthermore, embryos were cultured in HTF medium containing 100 nM of des-octanoyl 3 ghrelin (Phoenix Pharmaceuticals, Inc.), an analog lacking biological activity (6). For controls, embryos were cultured in HTF medium alone or containing 100 nM of ghrelin.

Embryonic development was monitored daily by phase-contrast microscopy (Olympus Corp.), and the rate of embryo development was assessed.

Differential labeling of inner cell mass (ICM) and trophectoderm (TE) nuclei

Embryos at the two-cell stage were cultured with 10 nM of ghrelin for 56 h, and the numbers of ICM and TE cells of each blastocyst were counted by the differential labeling technique using two polynucleotide-specific fluorochromes (propidium iodide and bisbenzimide; Hoechst 33342, Sigma) as described previously (31). After staining, the blastocysts were mounted on a glass slide, and the number of total, TE, and ICM cells in each blastocyst was counted under an epifluorescence microscope (Olympus Corp.).

Statistical analysis

To analyze the effect of ghrelin on embryo development, ordinal unpaired comparison *t* test and the analysis of sources of variation, *F* test, as well as the trend analysis (33–35) were performed on all of the possible pairs. The logarithms of observed four ghrelin concentrations are ordered from −1 (log [0.1 $_{nM}$]) to 2 (log [100 $_{nM}$]) with equal interval, 1. Thus, multiple comparisons of a series of observations at four different concentrations can be considered as those of ordered groups. In other words, further information about the nature of dependency induced by logarithmic concentration of ghrelin can be elucidated by using trend analysis. Considering linear ($df1 = 1$, $df2 = n - k - 1$), quadratic ($df1 = 2$, $df2 = n - k - 2$), cubic ($df1 = 3$, $df2 = n - k - 3$), and quartic ($df1 = 4$, $df2 = n - k - 4$), where n is a number of embryos used in each experiment and k is the number of experiments, trend analyses were performed. The *t* tests were performed with SPSS 10.1 (SPSS, Inc., Japan Inc.), and the calculations for the *F* tests and the trend analysis were performed with Excel 2001 (Microsoft Corp., Redmond, WA), according to published procedures (33).

The one-way ANOVA followed Fisher's protected least significant difference test was used to evaluate differences in ghrelin protein concentrations in plasma and uterine fluid, and the Mann-Whitney *U* test was performed for the comparison of the number of total blastomeres, ICM, and TE cells.

Results

Temporal expression of ghrelin and GHS-R mRNAs in mouse oocytes and preimplantation embryos

RT-PCR and nested PCR were performed to detect mRNAs for ghrelin and GHS-R in the mouse oocytes and early embryos at different stages (two-cell, four-cell, eight-cell, morula, blastocyst, and hatched blastocyst). Ghrelin and GHS-R mRNAs were detected in morula, blastocyst, and hatched blastocyst as 350-bp and 354-bp bands, respectively (Fig. 1). Two different sizes of ghrelin bands were obtained in placenta used as positive controls (Fig. 1). DNA sequencing of these bands (data not shown) revealed that the larger band was the expected proghrelin amplification product of 350 bp, whereas the lower band was a novel proghrelin fragment of 239 bp in length with a C-terminal truncation (missing 14th Gln of the mouse ghrelin and position 241–348 of the mouse proghrelin region; Ref. 36). Furthermore, the identity of the single GHS-R product was confirmed by DNA sequencing. As loading controls, no significant differences were observed in the intensities of α-globin and β-actin amplification products among oocytes and preimplantation embryos at different stages.

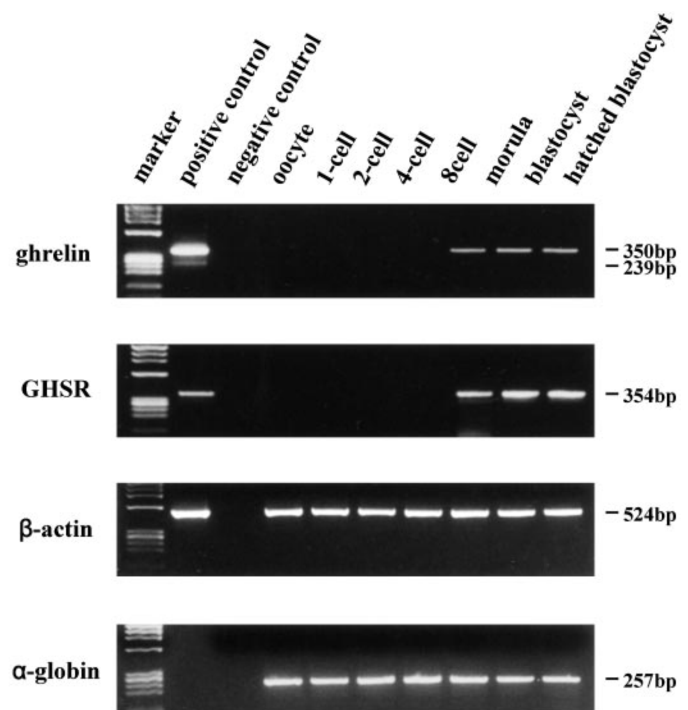

FIG. 1. RT-PCR detection of ghrelin and GHS-R mRNAs from mouse oocytes and preimplantation embryos. Fifteen oocytes and embryos at different stages were used for mRNA extraction. To compare amounts of the PCR product from the same number of actively transcribing genomes, the amount of cDNA for each PCR was corrected by the genome copies. Exogenous α-globin mRNA was added to each sample before mRNA extraction to evaluate the efficiencies of mRNA extraction and RT. For internal control, β-actin was amplified simultaneously in each PCR. Because of the low number of oocytes and preimplantation embryos under study, heminested PCR was needed to obtain optimal results. The expected 354-bp GHS-R PCR product is detected in morula, blastocyst, and hatched blastocyst stage embryos. No significant differences are observed in the signal intensities of α-globin and β-actin amplification products among oocytes and embryos at different stages. Experiments in the present study were performed three times on five separate pools of 15 oocytes and early embryos with reproducible results. The marker, $\phi \times 174$-Hae III digest; positive control for ghrelin, mouse placenta cDNA; positive control for GHS-R, mouse brain cDNA; negative control, without template cDNA.

Binding of fluorescent ghrelin to mouse preimplantation embryos

To confirm the expression of functional GHS-R in mouse preimplantation embryos, binding studies were performed using FAM-ghrelin. The clustered fluorescent signals were detected homogeneously in both ICM and TE cells at the blastocyst stage (Fig. 2, A–E), and the ratios of fluorescent pixels per embryo were saturated at 10 nM of FAM-ghrelin (Fig. 2B and Table 2). Embryos treated with 100 nM of FAM-ghrelin (Fig. 2C and Table 2) showed a similar level of fluorescent signals as compared with those treated with 10 nM of FAM-ghrelin (Fig. 2B and Table 2). In contrast, in four-cell stage embryos, no obvious signals were observed even in embryos treated with 100 nM of FAM-ghrelin (Fig. 2F). For controls, embryos incubated only with HTF medium also showed no signal (Fig. 2, E and H). Furthermore, no signals

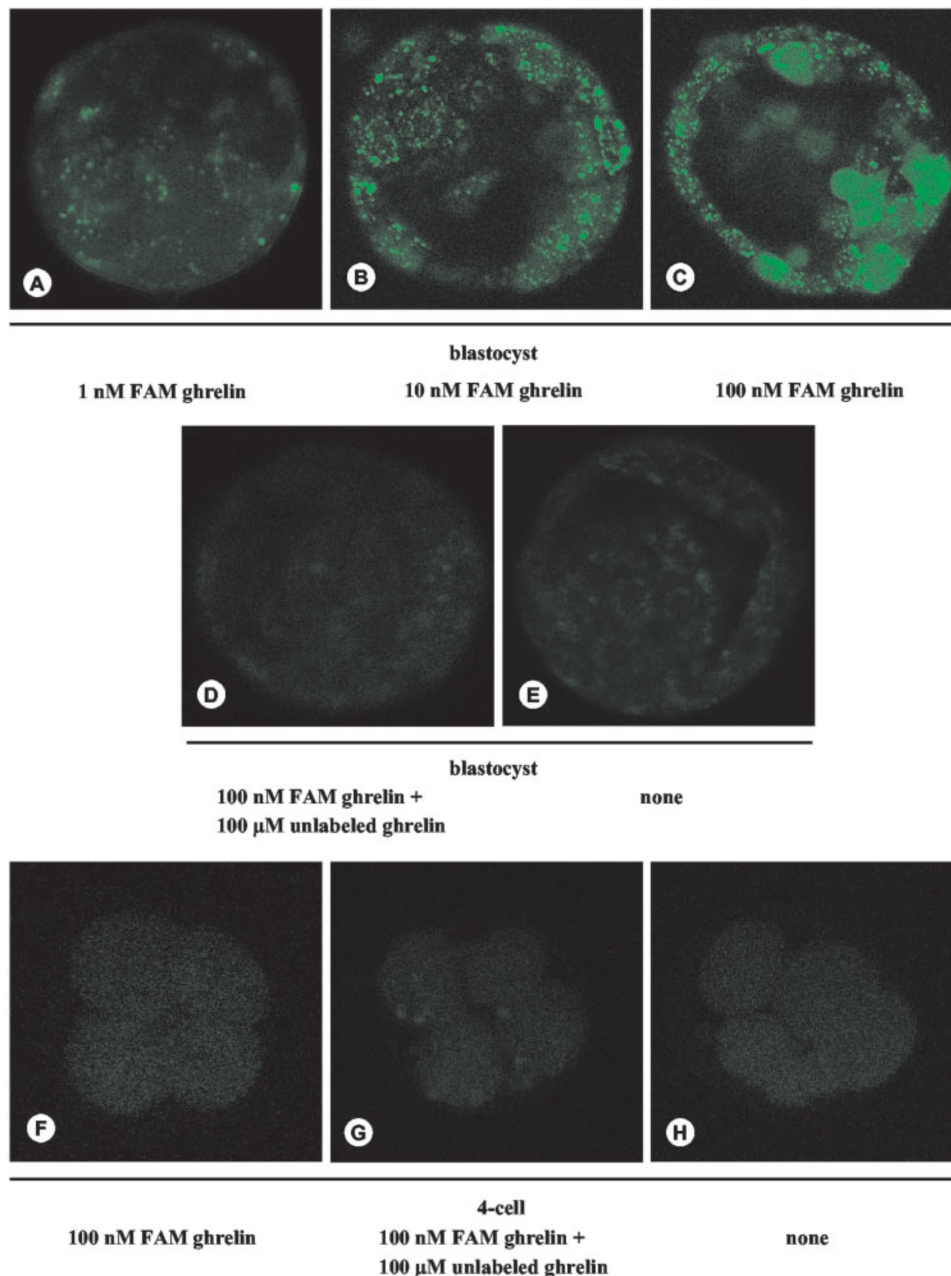

FIG. 2. Binding of fluorescent ghrelin to mouse preimplantation embryos. Shown are confocal images of optical sections of the following. A–E, Blastocyst stage embryos; F–H, four-cell stage embryos. Embryos were incubated with 1, 10, and 100 nM of FAM-ghrelin in HTF medium for 30 min at 37 C in 5% CO_2 in air and fixed in 4% paraformaldehyde. The clustered fluorescent signals of FAM-ghrelin are found homogeneously in both ICM and TE cells at 1.0 nM (A). The clustered fluorescent signals increase in 10 nM (B), but are saturated in 100 nM (C). Four-cell stage embryos lack fluorescent signals even at 100 nM (F). For controls of background fluorescence and nonspecific bindings, embryos were incubated only with HTF medium (E and H) and with a 100-fold excess of unlabeled ghrelin (D and G). The fluorescent signals in these controls are much weaker than specific signals in A–C. Confocal images were taken at magnification of ×63. Consistent signals were observed in at least three experiments in which a total of five four-cell and blastocyst stage embryos were surveyed.

TABLE 2. Ratio of fluorescent pixels obtained from confocal microscopic eight-bit images of mouse blastocyst

Ghrelin	Number of fluorescent pixels (A)	Number of pixels in whole embryo's area (B)	Ratio of pixels per embryo (A/B)
1 nM	7,959 ± 973	52,065 ± 357	0.1530 ± 0.0042
10 nM	27,147 ± 3,767	52,866 ± 2,710	0.5048 ± 0.1025
100 nM	38,221 ± 3,008	57,903 ± 2,260	0.6594 ± 0.0996

A total of five embryos were used in each concentration.

could be detected when embryos were incubated with the fluorescent ligand in the presence of a 100-fold excess of unlabeled ghrelin (Fig. 2, D and G).

Detection of ghrelin mRNA and protein in mouse endometrium

We hypothesize that embryos bearing GHS-R could be activated by ghrelin secreted by the uterus. RT-PCR was performed to detect ghrelin mRNA in the mouse uterus. Ghrelin mRNA was detected in uterus as a 394-bp band (Fig. 3a). As described above, the *lower band* corresponded to the truncated proghrelin fragment of 286 bp. Immunohistochemical staining was performed to detect ghrelin protein in the mouse endometrium. According to the sequence analysis, the *lower band* was a novel mouse ghrelin fragment, which corresponded to the rat des-Gln^{14}-ghrelin (36). The rat des-Gln^{14}-ghrelin was identified as a splice variant of ghrelin also with GH-releasing activity. However, the role of this novel form of mouse ghrelin in reproductive tract is unknown. At the protein level, the luminal and glandular epithelia of the endometrium were stained with the ghrelin antibody (Fig. 3b-A). The specificity of this immunoreactivity was demonstrated by the absence of staining in specimens incubated with nonimmunized serum (data not shown) and preabsorbed primary antiserum for ghrelin (Fig. 3b-B).

Determination of ghrelin level in the mouse uterine fluid

To further examine whether ghrelin is secreted by the endometrium, the levels of ghrelin in plasma and uterine fluid were measured using the ghrelin EIA. Plasma ghrelin concentrations were significantly increased in both nonpregnant and pregnant mice at 48 h after fasting as compared with nonfasted mice (both $P < 0.05$; Fig. 4A). There were no significant differences in plasma ghrelin concentrations between pregnant and nonpregnant mice (Fig. 4A). Similar results were obtained from uterine fluid samples (Fig. 4B). The ghrelin concentrations of uterine fluid were significantly increased in both nonpregnant and pregnant mice at 48 h after fasting as compared with nonfasted mice (both $P < 0.0001$; Fig. 4B). The level of ghrelin in uterine fluid of pregnant mice was slightly higher than that of nonpregnant mice, but the difference did not reach a significant level (Fig. 4B).

The effect of ghrelin on the development of preimplantation embryos in vitro

We hypothesized that increases of ghrelin in the uterine fluid of fasting animals could regulate early embryo development and determined the effects of ghrelin treatment on the *in vitro* development of mouse preimplantation

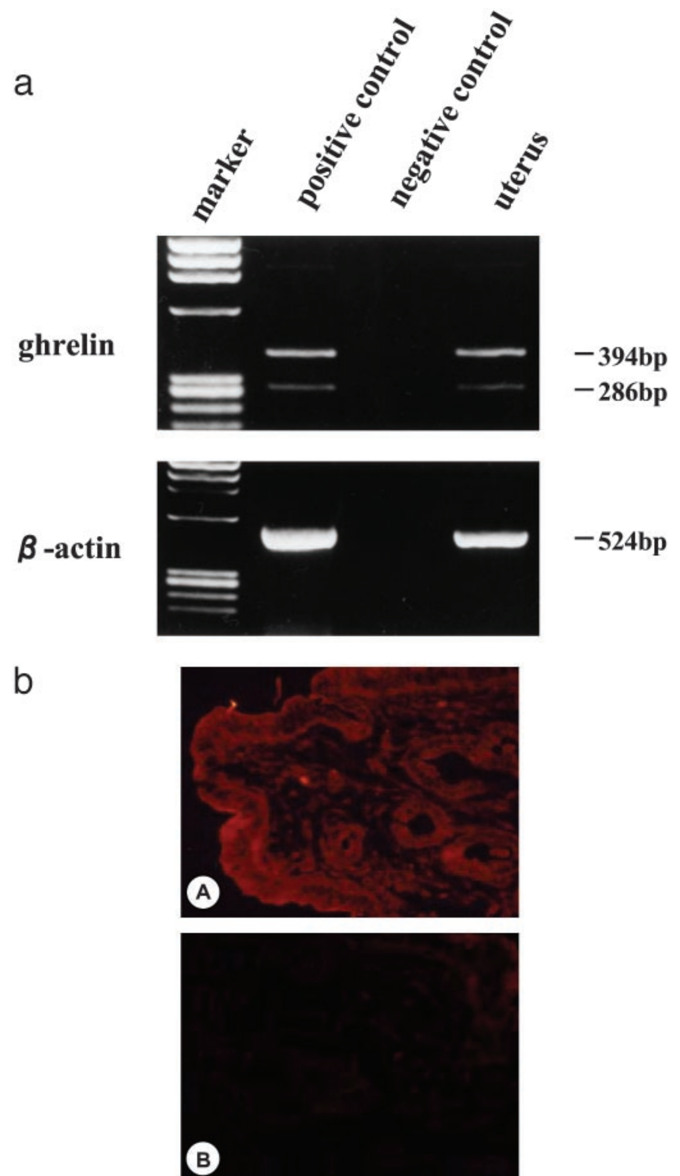

FIG. 3. a, RT-PCR detection of ghrelin mRNA from uterus of pregnant mice at d 4.5. Total RNA was extracted from uterus, and RT-PCR was performed. For internal control, β-actin was amplified simultaneously. Based on the DNA sequencing of ghrelin PCR products, the larger molecular weight band corresponded to an expected ghrelin amplification product of 394 bp, and a smaller 286-bp product corresponded to a transcript with a C-terminal truncation (missing 14th Gln of the mouse ghrelin and position 241–348 of the mouse proghrelin) is detected in uterus. Experiments in the present study were performed three times with reproducible results. The marker, $\phi \times 174$-Hae III digest; positive control, mouse placenta cDNA; negative control, distilled water. b, Immunofluorescence staining of ghrelin in mouse endometrium. Samples were fixed in 4% paraformaldehyde and stained using rabbit antighrelin serum with a dilution of 1:150 as primary antibodies and 1.0 μg/ml of goat antirabbit Cy3 fluorescein antibody as secondary antibodies. Immunoreactivity is detected in the luminal and glandular epithelia of endometrium (bA). Absorption with 50 μg/ml of ghrelin peptide before immunostaining abolished all positive staining (bB). Original magnification, ×200. Consistent staining was observed in at least 3 experiments in which a total of 10 mouse endometrium were surveyed.

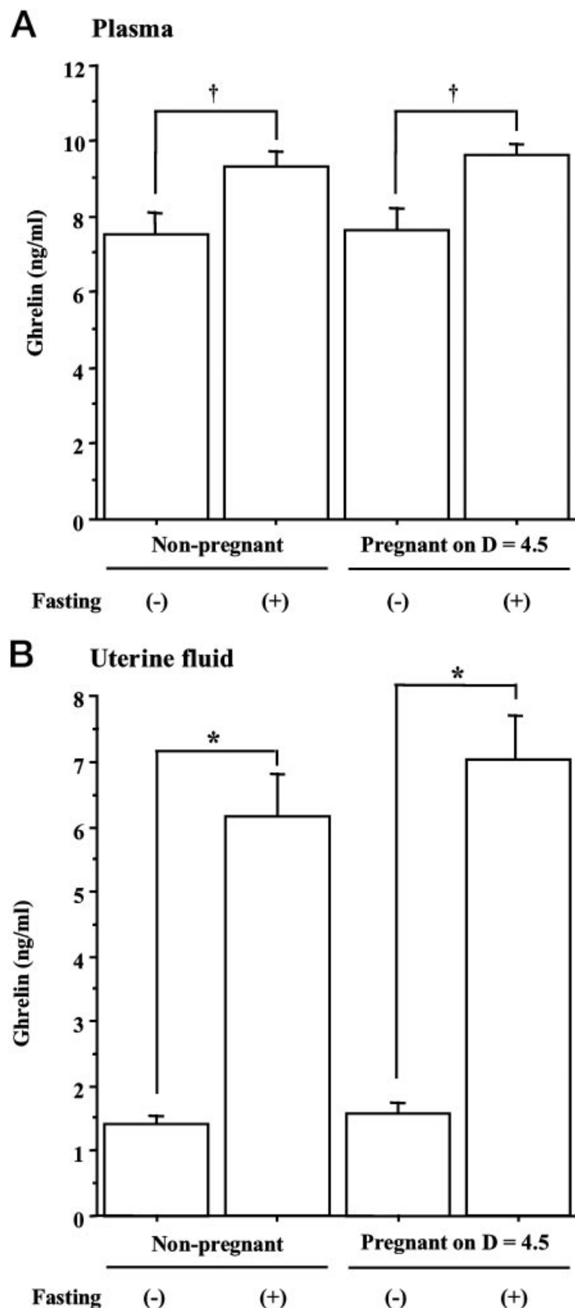

FIG. 4. The levels of ghrelin in mouse plasma (A) and uterine fluid (B) during early pregnancy. *Bars* represent mean ± SEM (n = 10). Nonpregnant, Nonpregnant mouse 9 wk of age at estrus; Pregnant on D = 4.5, pregnant mouse 9 wk of age at d 4.5; Fasting, 48 h of fasting (only access to water was allowed.) Each uterine fluid was obtained by flushing the uterine cavities with 10 μl of 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate buffer containing 0.6 TIU/ml of aprotinin and centrifuged at 2000 × *g* for 5 min. The plasma samples were obtained simultaneously. Ghrelin concentration was determined by EIA. Data were analyzed one-way ANOVA followed Fisher's protected least significant difference test. *, $P < 0.0001$; †, $P < 0.05$.

embryos. Two-cell stage embryos were cultured in the presence of 0.1, 1, 10, and 100 nM of rat ghrelin. In each experiment, 20–28 embryos were used in each group, consisting of six observations, and the experiment was repeated five times. Results of examination of a total of 123–159 embryos in each group were summarized in Fig. 5. Up to 36 h of culture, ghrelin treatment showed no effect on the development of preimplantation embryos to the morula stage. After 48 and 56 h of culture, 100 nM of ghrelin significantly inhibited the development of embryos from morula to blastocyst and from blastocyst to expanded blastocyst stage ($P = 0.02$ and 0.003 for none *vs.* 100 nM, respectively) with embryo development retarded at morula and blastocyst stages, respectively. After 72 h of culture, the rates of formation of hatched blastocyst from expanded blastocyst were significantly inhibited by 10 and 100 nM of ghrelin ($P = 0.007$ for none *vs.* 10 nM, and $P = 0.002$ for none *vs.* 100 nM). The slopes of observed ratio between the consecutive two points on the logarithmic concentrations of ghrelin were compared in each developmental stage. The absolute value of the slope between 1.0 and 10 nM of ghrelin was the highest for all the stages of embryos as well as for the culture periods, 48 h, 56 h, and 72 h (data not shown). Thus, a threshold value in the inhibitory effect of ghrelin existed between 1.0 and 10 nM. In addition, the observed P values of F test revealed that any developmental stages were not significant in either linearity, quadratic, cube, or quartic ($0.997 > P > 0.526$ for all developmental stages and at all culture periods). Thus, the nature of the inhibitory effect of ghrelin in the development of preimplantation embryos may be explained as a more complicated trend.

To confirm the specificity of the inhibitory effect of ghrelin on the preimplantation embryos, the effects of an antagonist for GHS-R, [D-Lys-3]GHRP-6, and a nonbioactive form of ghrelin, des-octanoyl 3 ghrelin, were examined by an additional five sets of experiments. Two-cell stage embryos were cultured with 1) HTF medium alone; 2) 100 nM of ghrelin; 3) 10 mM of [D-Lys-3]GHRP-6; 4) 100 nM of ghrelin and 10 mM of [D-Lys-3]GHRP-6; and 5) 100 nM of des-octanoyl 3 ghrelin. In each experiment, 22–28 embryos were used in each group, consisting of 6 observations, and the experiment was repeated 5 times. A total of 145–168 embryos were tested in each group, and the results were summarized in Fig. 6. The inhibitory effects of ghrelin on the development of embryos from morula to the blastocyst, blastocyst to expanded blastocyst, and expanded blastocyst to hatched blastocyst stage were significantly blocked by treatment of [D-Lys-3]GHRP-6 ($P < 0.008$, $P < 0.001$, and $P < 0.000$, *vs.* 100 nM of ghrelin, respectively). [D-Lys-3]GHRP-6 alone and des-octanoyl 3 ghrelin showed little effect in the suppression of embryo development.

The effect of ghrelin on the regulation of cell numbers in cultured mouse blastocyst

The numbers of total, TE, and ICM cells of blastocyst after 56 h of culture with or without ghrelin are summarized in Fig. 7. Blastocysts cultured with 10 nM of ghrelin had a significantly lower total cell number, as compared with blastocysts cultured in HTF medium alone. The decrease in the total cell number of ghrelin-treated blastocysts resulted from inhibition of the proliferation of both ICM and TE cells, and

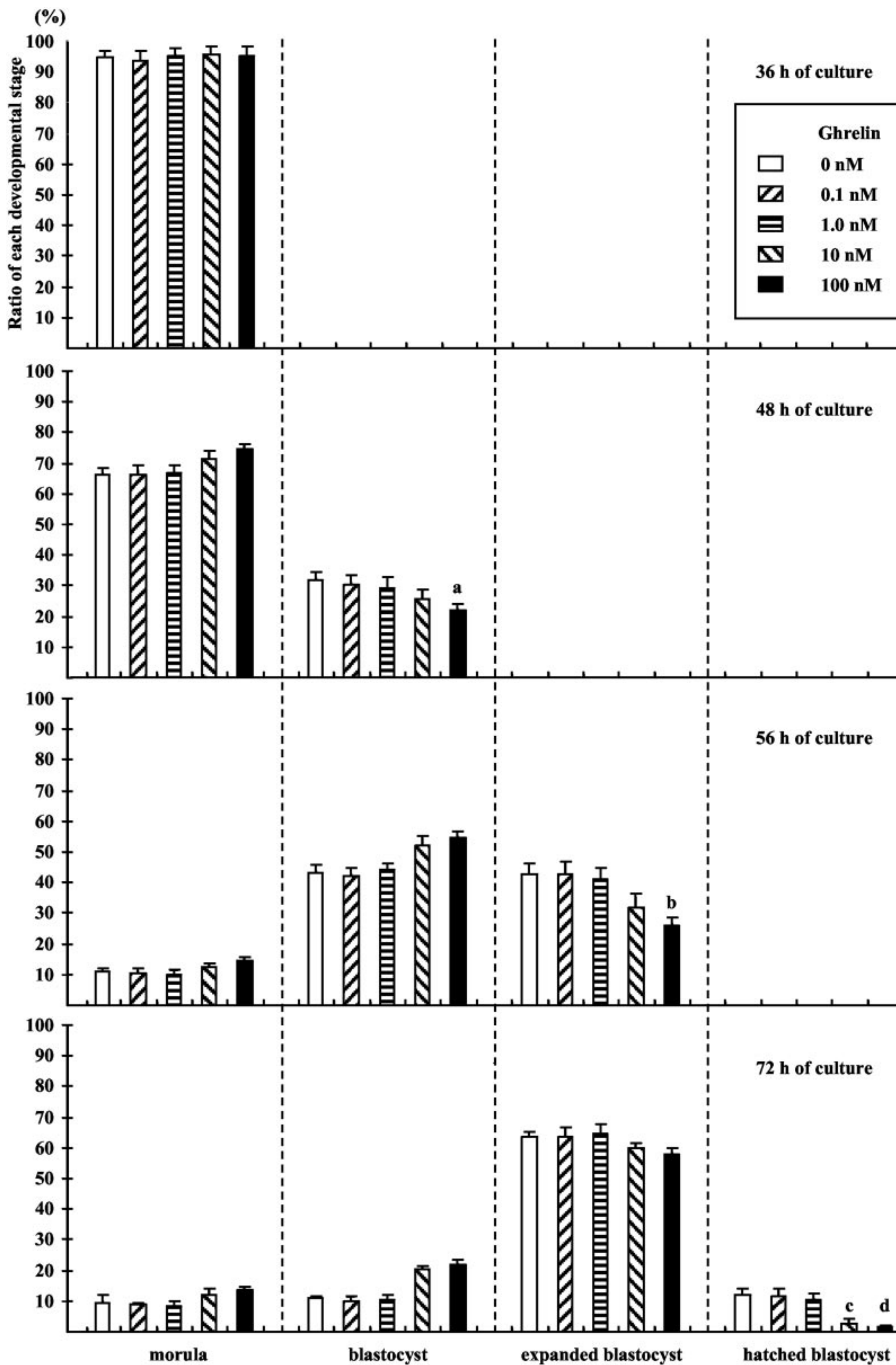

FIG. 5. The dose-dependent effects of ghrelin on the *in vitro* development of mouse preimplantation embryos. In each experiment, 20–28 embryos were used in each group, consisting of 6 observations, and the experiment was repeated 5 times. A total of 123–159 embryos were examined in each group; 0 nM = 159, 0.1 nM = 120, 1.0 nM = 123, 10 nM = 124, 100 nM = 130. Values are mean ± SEM. The data were analyzed by unpaired comparison *t* test and the analysis of sources of variation, *F* test, as well as the trend analysis. a, $P = 0.02$ for none *vs.* 100 nM; b, $P = 0.003$ for none *vs.* 100 nM; c, $P = 0.007$ for none *vs.* 10 nM; d, $P = 0.002$ for none *vs.* 100 nM.

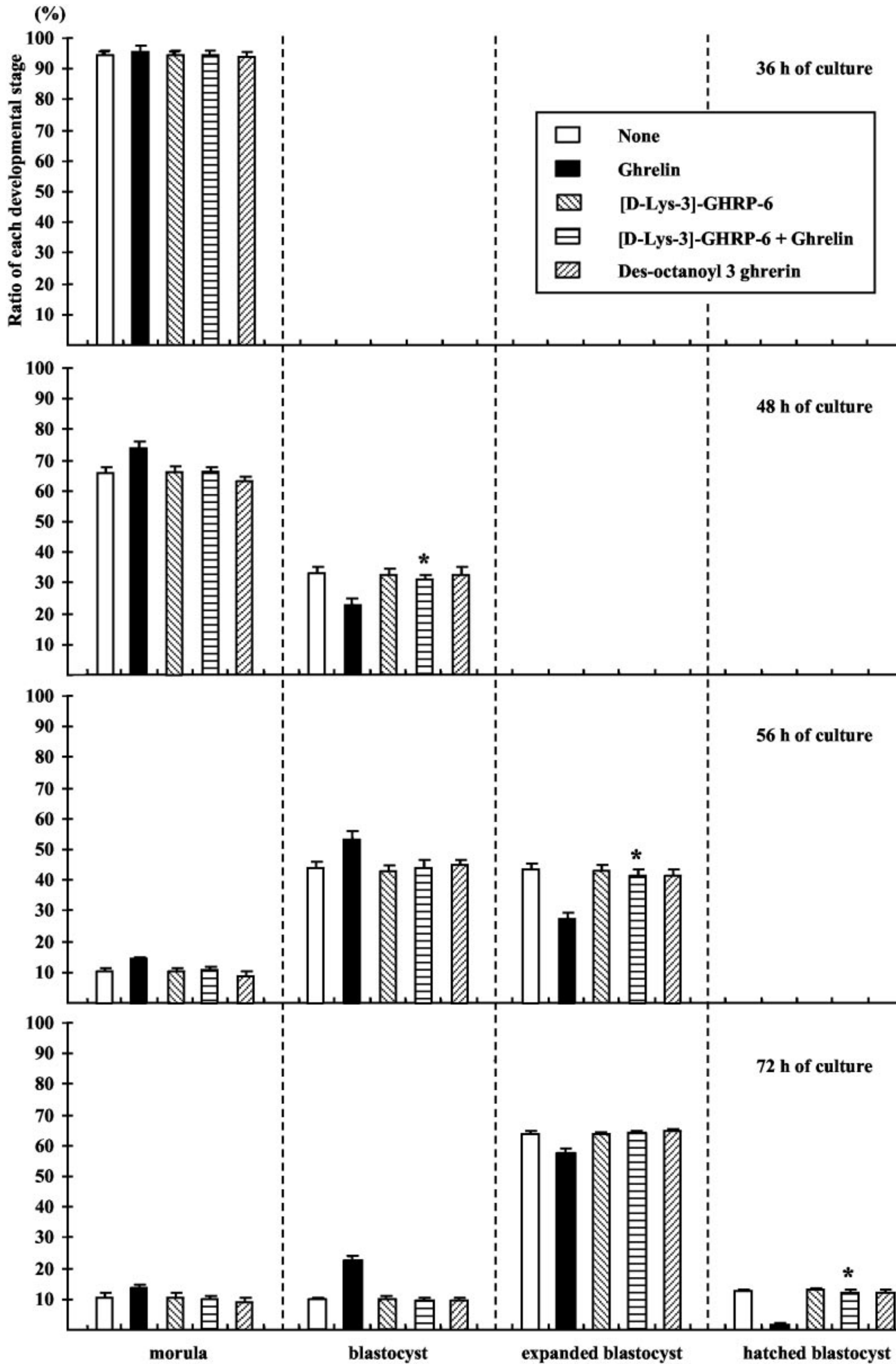

FIG. 6. The effects of an antagonist for GHS-R, [D-Lys-3]GHRP-6 and a nonbioactive form of ghrelin, des-octanoyl 3 ghrelin. In the additional five sets of experiments, embryos were cultured in: 1) HTF medium alone; 2) 100 nM of ghrelin; 3) 10 mM of [D-Lys-3]GHRP-6; 4) 100 nM of ghrelin and 10 mM of [D-Lys-3]GHRP-6; and 5) 100 nM of des-octanoyl 3 ghrelin. In each experiment, 22–28 embryos were used in each group, consisting of exactly 6 observations, and the experiment was repeated 5 times. Between 145 and 168 embryos were tested in each group; 1) 168, 2) 145, 3) 145, 4) 159, and 5) 159. None, HTF medium only. Values are mean ± SEM. The data were analyzed by unpaired comparison *t* test and the analysis of sources of variation, *F* test. *, $P < 0.01$, *vs.* 100 nM of ghrelin.

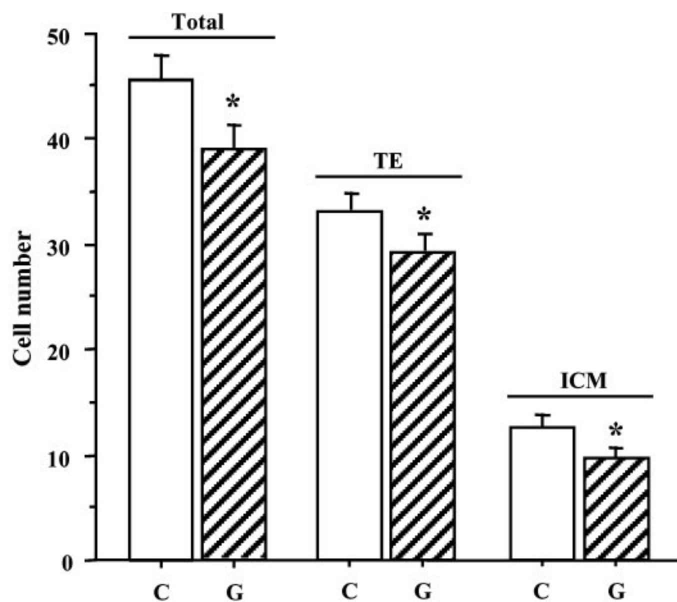

FIG. 7. The effect of ghrelin on the number of total cells (Total), ICM cells, and TE cells in 56-h cultured blastocysts. Values are mean ± SEM of blastocysts cultured in 10 nM of ghrelin (G; n = 30) and blastocysts cultured in HTF medium alone (C; n = 30). Data were analyzed by Mann-Whitney *U* test. *, $P < 0.05$, significantly different from corresponding control.

the inhibitive effect was equally observed in TE cells and ICM cells (both $P < 0.05$).

Discussion

In the present study, we demonstrate the temporal expression of ghrelin and GHS-R mRNAs in mouse oocytes and preimplantation embryos. Both ghrelin and GHS-R mRNAs were expressed in mouse morula, blastocyst, and hatched blastocyst stage embryos. Using binding assays, fluorescent-labeled ghrelin could bind to both ICM and TE cells at blastocyst stage as clustering patterns, suggesting the existence of receptor-ligand complexes (37, 38). In contrast, four-cell stage embryos lacked specific fluorescent signals. The results of both RT-PCR and binding assay also suggest that four-cell stage embryos do not express functional receptors. Thus, through the receptor-mediated process, exogenously supplemented ghrelin could be taken into embryos expressing the GHS-R. Ghrelin protein was expressed in the epithelia of endometrium as shown by immunohistochemistry. Furthermore, ghrelin mRNA was expressed in uterus of early pregnant mice. Thus, these data strongly suggested that ghrelin is produced by endometrial epithelium.

We further confirmed whether ghrelin was secreted during early embryogenesis by the reproductive tract of mouse. Ghrelin could be detected in uterine fluid, and the level was significantly increased in fasting mice as compared with those with free access to foods. These findings suggest that ghrelin is produced and secreted from endometrial epithelium and may regulate the function(s) of preimplantation embryo during its development in a paracrine/autocrine manner.

Accumulated evidence indicates that a number of growth factors and cytokines contribute in a paracrine and/or autocrine fashion to the rate of embryo development, the proportion of embryos developing to the blastocyst stage, the cell number in the blastocyst, energy metabolism, and apoptosis (reviewed in Ref. 39). Supplementation of culture medium with exogenous growth factors and cytokines affects the development of preimplantation embryos via paracrine pathways (reviewed in Ref. 39). Although much of the work on the stimulating effects of growth factors and cytokines on preimplantation embryos has been carried out using culture conditions, there have been only a few reports concerning inhibitory factors. TNF-α was reported to inhibit cell proliferation in blastocyst and induce apoptosis in the ICM (40–44). Furthermore, TNF-α decreases the ability of embryos to differentiate into fetuses after implantation (44). Interferon-γ inhibited blastocyst formation and trophoblast outgrowth after attachment *in vitro* (45, 46). The data obtained from the present study have shown that the addition of ghrelin to mouse embryo culture media can inhibit preimplantation embryo development from two-cell stage embryo to the blastocyst, fully expanded blastocyst, and hatched blastocyst *in vitro* in a dose-dependent manner. This effect was blocked by an antagonist for GHS-R, [D-Lys-3]GHRP-6. Therefore, ghrelin is one of the inhibitory factors for the development of preimplantation embryos. Although ghrelin could inhibit the development of embryos as a paracrine factor, treatment of antagonist for GHS-R alone showed little effect on the development of embryos. Because the levels of ghrelin in the embryo culture medium were under the sensitivity of assay detection (data not shown), the level of ghrelin secreted from embryo itself may be insufficient for inducing inhibitory effects. Thus, further studies will be required to elucidate the autocrine mechanism within mouse preimplantation embryos.

It is well known that disorders in nutritional status can disrupt the complex interplay of gonadotropins and gonadal hormones, which are essential for fertility. Suppression of pulsatile LH secretion has been reported after fasting or food restriction in mammals, including rodents and humans (47–55). Fasting-induced suppression of LH is considered to be a result of reduced secretion of GnRH from the hypothalamus, because fasted animals show LH pulses similar in quantity and magnitude to fed ones when administrated exogenous GnRH (53, 56–60). Previous reports have demonstrated that plasma ghrelin levels rose in response to food restriction or fasting as well as aging (12, 21–24, 61). Thus, ghrelin may act as a peripheral factor to avoid the excess metabolic demands imposed by reproduction during insufficient nutrient intake.

In the present study, fasting for 48 h led to increased secretion of ghrelin into uterine fluid as observed in plasma samples. The inhibiting effect of ghrelin on embryo development was observed when the concentration of ghrelin exceeded to threshold between 1.0 nM and 10 nM. The binding assay also showed that the levels of fluorescent signals in embryos were saturated at 10 nM of FAM-ghrelin. The concentration of ghrelin in uterine fluid of fasting mice at d 4.5 of pregnancy was determined to be 7.02 ± 0.70 ng/ml (2.12 ± 0.21 nM), a level found to regulate embryo development.

At the blastocyst stage, the embryo consists of two types of cell lineage, TE and ICM cells. The TE cells are necessary

for implantation and subsequent formation of the placenta and extraembryonic membranes. The ICM cells form all three germ layers and all tissues of the embryo, as well as extraembryonic membranes. Thus, cell numbers in the TE, in the ICM, or in both cell populations of blastocyst are the indicators of embryo growth and viability (62). Although TNF-α treatment predominantly suppresses the ICM lineage (40–44), ghrelin decreases the total cell number of blastocysts as a result of reduction of the numbers of both ICM and TE cells. These differences may be caused by the differential expression pattern of specific receptors for TNF-α and ghrelin in blastocyst. The TNF-α receptors were shown to be localized mainly in ICM (41), whereas fluorescent ghrelin was detected in both ICM and TE cells.

Recently, some GHS were reported to inhibit proliferation of thyroid, breast, and lung cancer cell lines as assessed by thymidine incorporation and cell proliferation (27–29). Among the GHS, ghrelin was shown to inhibit thymidine incorporation and proliferation of a breast cancer cell line at concentrations close to its binding affinity (29); however, several conflicting data have been reported in other cell lines. Ghrelin simulated proliferation of prostate cancer cell line (63) and cardiomyocyte cell line (64).

In conclusion, we demonstrate the temporal expression of ghrelin and GHS-R mRNAs in mouse preimplantation embryos. Both ghrelin and GHS-R mRNAs were detected after morula stage embryos. Ghrelin was produced and secreted from reproductive tracts, and the level of ghrelin was elevated with fasting. Furthermore, high levels of ghrelin could inhibit the development of mouse preimplantation embryos through its specific receptor, GHS-R. These observations strongly suggest that ghrelin could inhibit the development of preimplantation embryos under malnutritional status.

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