volume and the concentration of IL-8. After collection of the culture media, the cultured cells were homogenized, and the amount of total protein in the homogenized cells was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Data of IL-8 content in the culture media were normalized against the total protein content of the cell lysates.

In vitro migration assay

In vitro migration assay was performed in 24-well plates containing Transwell permeable supports with an 8-μm polycarbonate membrane (Costar, Cambridge, MA) for vCTs as previously described (2). EEC-SNs that had or had not been stimulated by 0.1, 1, and 10 ng/ml IL-1β for 24 h [IL-1β (0.1, 1, 10 ng/ml) EEC-SN and control EEC-SN, respectively] or FBS-free medium with or without IL-8 (100 pg/ml) was added to the lower chambers. In the experiments using the neutralizing antibody, control EEC-SN and EEC-SN that had been stimulated by 1 ng/ml IL-1β for 24 h [IL-1β (1 ng/ml) EEC-SN] were preincubated for 1 h with 1 μg/ml of IL-8Ab or isotype control mIgG and plated in the lower chambers. Cultured vCTs were plated at a density of 2×10^5 cells/well in the upper chamber of the Transwell membranes, which contained 100 μl FBS-free DMEM/F12, and were incubated for 72 h at 37 C in a 5% CO_2 atmosphere. After the incubation, the upper surface of the membranes was gently cleansed with a cotton swab. Then the cells that had migrated through the pores were fixed with acetone/methanol and stained with hematoxylin and eosin. The filter was gently cut from the chamber, and the migrated cells were counted from the underside of the filter. The number of vCTs that migrated across the filters was counted in 10 randomly selected high-power fields (HPFs) per filter under the light microscope.

Measurement of vCT number

To measure the cell number, we used the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) as we previously described (28).

The cultured medium was removed from vCTs and replaced with fresh medium supplemented with antibiotics 24 h before treatment. Cultured vCTs were treated with control and IL-1β (0.1, 1, 10 ng/ml) EEC-SN or FBS-free medium with or without 100 pg/ml IL-8. In the experiments using neutralizing antibody, control and IL-1β (1 ng/ml) EEC-SN were preincubated for 1 h with 1 μg/ml IL-8Ab or isotype control mIgG before the treatment of vCTs was started. After 72 h incubation, CCK-8 assay was performed.

In the CCK-8 assay, CCK-8 solutions containing the tetrazolium salt WST-8 were added and incubated at 37 C for an additional 2 h. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product in culture medium. The amount of formazan, which is directly proportional to the number of living cells, was evaluated by measuring the OD at 450 nm in the DigiScan microplate reader (ASYS Hitech).

RT-PCR of *CXCR1* and *CXCR2* mRNA

RT-PCR was performed as reported previously (29). Total RNA was extracted from vCTs and PLs using the RNAeasy minikit (QIAGEN, Hilden, Germany). Reverse transcription was performed using ReverTra Ace-α (Toyobo, Tokyo, Japan). One microgram of total RNA was reverse transcribed in a total volume of 20 μl, and cDNA was amplified using oligonucleotide primers based on the human *CXCR1* and *CXCR2* sequences. PCR was performed using the ReverTra Dash kit (Toyobo) according to the manufacturer's instructions. *CXCR1* primers (sense, 5′-TACTGTTGGACACACCTGGC-3′; antisense, 5′-TAGACATCAGTGACGGAGCG-3′) were designed to amplify a 294-bp fragment. *CXCR2* primers (sense, 5′-CACAGTGAAGACATCGGTGG-3′; antisense, 5′-AGGGATTCTGGTTCACATGG-3′) were designed to amplify a 246-bp fragment. The expression levels of *CXCR1* and *CXCR2* mRNA were normalized to *GAPDH* mRNA, which was used as an internal control and as a loading control. Human *GAPDH* primers (Toyobo) were designed to amplify a 452-bp fragment. The PCR conditions for *CXCR1* consisted of 35 cycles at 98 C for 10 sec, 60 C for 4 sec, and 74 C for 12 sec. The PCR conditions for *CXCR2* consisted of 35 cycles at 98 C for 10 sec, 60 C for 4 sec, and 74 C for 12 sec. PCR products were purified using the QIAEX II gel extraction kit (QIAGEN), and their sequence identities confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis

Data were evaluated using ANOVA with *post hoc* analysis using Fisher's protected least-significance difference test. $P < 0.05$ were accepted as significant.

Results

Both basal and IL-1β-induced IL-8 levels of culture media are higher in EECs than ESCs

Both ESCs and EECs have been reported to produce IL-8 in response to IL-1 (24, 25, 30). IL-1 has two bioactive ligands, IL-1α and IL-1β, possessed of similar biological effects (31). Therefore, in the present study, we used IL-1β as a representative of IL-1 ligands, and compared IL-8 production in response to IL-1β between ESCs and EECs. As shown in Fig. 1, both the basal IL-8 levels and the IL-1β-induced IL-8 levels were higher in EEC-SN than in ESC-SN. The basal IL-8 level of EEC-SN was 72-fold higher than that of ESC-SN. The IL-8 level of media in EECs stimulated by 1 ng/ml IL-1β was 5 times higher than that of ESCs. Based on these results, we chose to use the EEC-SN in all subsequent experiments to evaluate the effects of endometrial IL-8 on first trimester vCT.

Migration of first-trimester vCTs is stimulated by EEC-SN treated with IL-1β via IL-8

To study the chemotactic effects of EEC-derived IL-8 on first-trimester vCTs, *in vitro* migration assay was performed. As illustrated in Fig. 2A, more vCTs migrated to IL-1β (0.1, 1, 10 ng/ml) EEC-SN than control EEC-SN. The effect appeared to be maximal at 1 ng/ml. Recombinant IL-8 also promoted the chemotaxis of

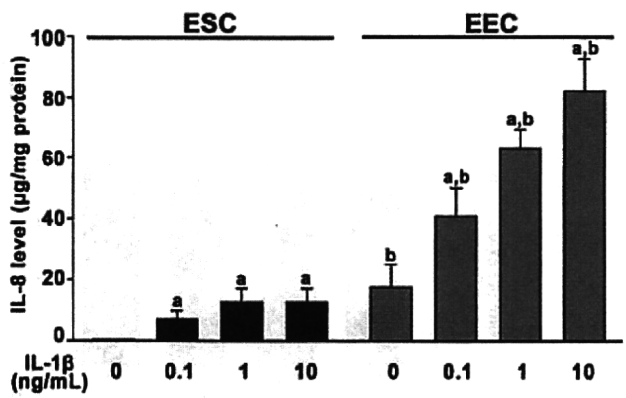

FIG. 1. IL-1β-stimulated IL-8 secretion from ESCs and EECs. Human ESCs (n = 10) and EECs (n = 10) were cultured in FBS-free media with different doses of IL-1β for 24 h. The supernatants were collected and assayed for IL-8 by ELISA. Values are normalized to the total protein content of the cell lysate. The values (total IL-8 content of the supernatants/total protein content of the cell extract, micrograms per milligram protein) are presented as the mean ± SEM of the combined data of separate experiments using different ESC and EEC preparations. a, $P < 0.05$ when compared with the same cell type without IL-1β stimulation; b, $P < 0.05$ when compared with ESCs stimulated by the same dose of IL-1β.

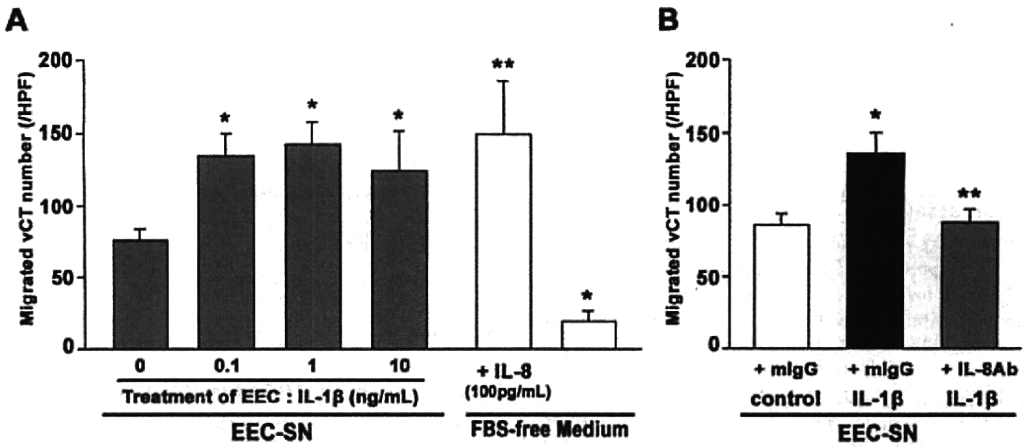

FIG. 2. Stimulatory effect of EEC-SN treated with IL-1β on migration of first-trimester vCTs through IL-8. *In vitro* migration assay was performed to determine whether migration of human first-trimester vCTs was affected by endometrial IL-8 expression. Cultured vCTs were plated at a density of 2×10^5 cells in the upper chambers of Transwell membranes for 72 h. After incubation, vCTs that had migrated through the pores were fixed and counted. The number of vCTs that had migrated across the filters was counted in 10 randomly selected HPFs per filter under the light microscope. Values represent the cell number per HPF. A, EEC-SN that had or had not been treated with IL-1β for 24 h (IL-1β EEC-SN and control EEC-SN, respectively) or FBS-free media with or without IL-8 were plated in the lower chambers. Values are the mean ± SEM of the combined data from six independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN; **, $P < 0.05$ when compared with FBS-free media without IL-8. B, Control EEC-SN and EEC-SN that had been treated with 1 ng/ml of IL-1β for 24 h [IL-1β (1 ng/ml) EEC-SN] were preincubated for 1 h with 1 μg/ml of anti-IL-8Ab or isotype control mIgG and plated in the lower chambers. Values are the mean ± SEM of the combined data from 10 independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN + mIgG; **, $P < 0.05$ when compared with IL-1β (1 ng/ml) EEC-SN + mIgG.

vCTs. In contrast, immunoneutralization with IL-8Ab abolished the chemotactic activity of IL-1β (1ng/ml) EEC-SN (Fig. 2B).

EEC-SN treated with IL-1β yielded higher number of first-trimester vCTs via IL-8

To study the effects exerted by EEC-derived IL-8 on number of first-trimester vCTs, we used CCK-8 assay. As illustrated in Fig. 3A, when compared with control EEC-SN, IL-1β (0.1, 1, 10 ng/ml) EEC-SN up-regulated the vCT number. Recombinant IL-8 also increased in the vCT number. The effects of IL-1β (1ng/ml) EEC-SN were eliminated by immunoneutralization with IL-8Ab (Fig. 3B).

CXCR1 and *CXCR2* mRNA are expressed in first-trimester vCTs

To examine the expression of IL-8 receptors in first-trimester vCTs, RT-PCR was performed using primers specific to *CXCR1* and *CXCR2* (receptors for IL-8). A sample of PLs was used as a positive control for IL-8 receptor-expressing cells (32–35). As demonstrated in Fig. 4, mRNAs of IL-8 re-

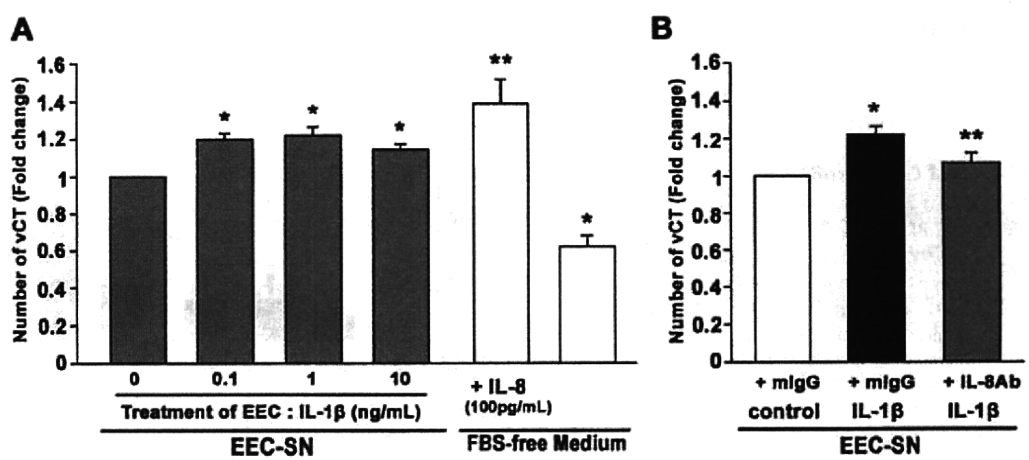

FIG. 3. Stimulatory effect of EEC-SN treated with IL-1β on increase in number of first-trimester vCTs through IL-8. The CCK-8 assay was performed to determine whether number of human first-trimester vCTs was affected by endometrial IL-8 expression. Before the assay, vCTs were treated for 72 h. A, vCTs were treated with control EEC-SN and IL-1β (0.1, 1, and 10 ng/ml) EEC-SN or FBS-free media with or without 100 pg/ml of IL-8. The values represent the ratio of the cell number relative to control EEC-SN. Values are the mean ± SEM of the combined data from seven independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN; **, $P < 0.05$ when compared with FBS-free media without IL-8. B, Control EEC-SN and IL-1β (1 ng/ml) EEC-SN were preincubated for 1 h with 1 μg/ml of IL-8Ab or mIgG. The values represent the ratio of the cell number relative to control EEC-SN with mIgG. Values are the mean ± SEM of the combined data from nine independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN + mIgG; **, $P < 0.05$ when compared with IL-1β (1 ng/ml) EEC-SN + mIgG.

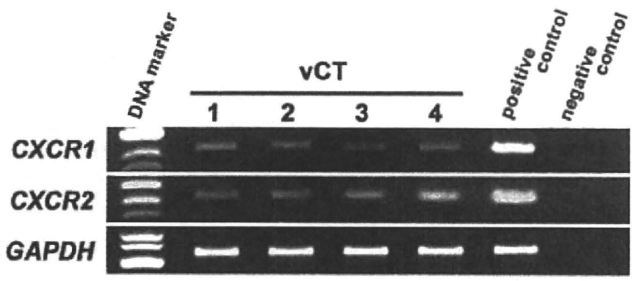

FIG. 4. Expression of IL-8 receptors *CXCR1 and CXCR2 mRNA in human first-trimester vCTs. Total RNA isolated from human first-trimester vCTs was reverse transcribed and amplified by PCR using primers for CXCR1 and CXCR2. Amplification of the internal control GAPDH was used to ensure RNA quality and as a loading control. The results are from four vCT samples (vCT 1–4) of different individuals. Gestational age of the vCT samples were; vCT1, 6 wk; vCT2, 6 wk, vCT3, 7 wk, vCT4, 8 wk. DNA marker, φX174/HinfI; positive control, peritoneal leukocytes; negative control, water without cDNA.*

ceptors *CXCR1* and *CXCR2* mRNA were expressed in first-trimester vCTs.

Discussion

In the present study, we demonstrated that IL-1β induced the production of IL-8 from endometrial cells and that endometrial cell-derived IL-8 promoted migration of first-trimester vCTs and yielded higher number of vCTs.

IL-1 is produced by human embryo and first-trimester vCTs (8, 9) and is thought to play a regulatory role in human embryo implantation. It has been reported previously that IL-1 up-regulates integrin-β3, a marker of uterine receptivity in endometrial epithelial cells (12). Furthermore, IL-1β can induce the expression of prostaglandins (36) and LIF (37), factors that are important for the implantation process (6), in human endometrial and decidual cells. Our findings that IL-1β stimulated migration of first-trimester vCTs and increased the cell number via endometrium-derived IL-8 suggest a novel function for IL-1β and extend the notion that IL-1β is a pivotal and multifunctional factor in human embryo implantation.

The CXC chemokine IL-8 participates in the migration of leukocytes such as neutrophils and T lymphocytes (20, 21, 38–41). As well as being able to encourage the accumulation of leukocytes, uterine IL-8 is thought to have unique roles in endometrial angiogenesis, apoptosis, proliferation, and differentiation (42). These events are crucial to preparing the endometrium for implantation. Combined with the promotive effects on migration of first-trimester vCTs and maintenance of the number of live cells in the present study, IL-1β-induced secretion of IL-8 may orchestrate the implantation process and therefore affect both the endometrium and trophoblast. As a note, because IL-8 is secreted by first-trimester extravillous trophoblast cell line HTR8 (43) and its secretion is enhanced by IL-1 in third-trimester trophoblast cells (44), IL-1 secreted by first-trimester vCTs may induce IL-8 secretion from vCTs themselves as well as endometrial cells. IL-1, therefore, might be involved in implantation via trophoblast-derived IL-8. However, in the present study, we did not observe any difference between cell-free IL-1β EEC-SN and FBS-free medium without any additions and between control EEC-SN with IL-8Ab and control EEC-SN with isotype mIgG on the migration and cell number of vCTs (data not shown). Further studies are warranted to elucidate the detail mechanism.

The current study demonstrated that both basal and IL-1β-induced IL-8 levels were much higher in EEC-SN than ESC-SN. The implication for this difference is not clearly understood at the moment. However, it is interesting to note that our recent study has demonstrated that CXCL11, which stimulates migration of first-trimester vCTs, is also inducible in epithelial cells but not stromal cells (2). It can therefore be speculated that like CXCL11, epithelium-derived IL-8 is involved in a relatively early-phase of implantation, assuming that the endometrial epithelium is an important component in initiating the molecular interactions between embryo and endometrium (2, 45).

Migration of first-trimester trophoblast cells needs to be a tightly regulated process for successful implantation and for circumventing undesirable complications of pregnancy such as poor fetal growth or preeclampsia. The chemokines CX3CL1, CCL14, CCL4, CXCL9, CXCL10, and CXCL11 promote the migration of trophoblast cells into the endometrium (1, 2). Furthermore, decidual natural killer cell-derived IL-8 promotes the invasion of first-trimester extravillous trophoblast cells into the decidua (20). Our findings are interesting in that trophoblast may control its own activity through cross talk with EECs. Collectively, the molecular mechanism that fine-tunes trophoblast migration is believed to work in a spatiotemporally specific manner.

A previous study demonstrated that the supernatants of first-trimester primary decidual cells inhibit the proliferation of trophoblast cell line BeWo cells, whereas those of BeWo cells and first-trimester primary trophoblast cells promote the proliferation of decidual cells (46). These findings indicate that a regulatory loop to control the growth of fetal and maternal cells exists at the fetomaternal interface. Thus, cross talk between trophoblast and endometrium seems to be important for maintenance of trophoblast cell numbers. The studies on the inhibitory role of IL-1β in the proliferation of trophoblast cell lines BeWo cells and JAR cells (47, 48) imply a possibility that IL-1β is one of the regulating factors in the growth of placenta. In light of our findings that IL-8 derived from IL-1β-stimulated EECs could yield a higher number of vCTs, it can be speculated that endometrial IL-8 may act as one of the survival factors for first-trimester vCTs and facilitate placental growth in early pregnancy. Thus, not only IL-1β but also subsequent IL-8 may participate in the regulatory loop of placental growth at the fetomaternal interface.

In summary, we have shown that IL-1β is able to induce secretion of IL-8 from EECs and that EEC-derived IL-8 is able to stimulate the migration of human first-trimester vCTs and yield a higher number of vCTs. These findings suggest that human vCTs may regulate their own status via IL-8 secreted by IL-1β-stimulated EECs to accomplish successful implantation.

Acknowledgments

Address all correspondence and requests for reprints to: Yutaka Osuga, M.D., Ph.D., Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: yutakaos-tky@umin.ac.jp.

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human reproduction

Reproductive biology

Progesterone decreases bone morphogenetic protein (BMP) 7 expression and BMP7 inhibits decidualization and proliferation in endometrial stromal cells

Ako Kodama, Osamu Yoshino, Yutaka Osuga[1], Miyuki Harada, Akiko Hasegawa, Kahori Hamasaki, Masashi Takamura, Kaori Koga, Yasushi Hirota, Tetsuya Hirata, Yuri Takemura, Tetsu Yano, and Yuji Taketani

Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

[1]Correspondence address. Tel: +81-3-3815-5411; Fax: +81-3-3816-2017; E-mail: yutakaos-tky@umin.ac.jp

BACKGROUND: Regulation of decidualization is decisive for proper implantation and the establishment of pregnancy. Recent studies have suggested that several bone morphogenetic proteins (BMPs) play physiological roles in reproduction. In the present study, we examined the expression of BMP7 in the endometrium and the effect of BMP7 on decidualization and proliferation of endometrial stromal cells (ESC).

METHODS: The gene expression of BMP7 in endometrial tissues collected from women with regular menstrual cycles was determined and the effect of ovarian steroid hormones on BMP7 gene expression was investigated in cultured ESC. The effect of BMP7 on the decidualization of ESC was determined by measuring the gene expression and protein secretion of insulin-like growth factor binding protein 1 (IGFBP1), a marker of decidualization. The effect of BMP7 on the proliferation of ESC was examined by the bromodeoxyuridine (BrdU) incorporation assay.

RESULTS: The gene expression of BMP7 in endometrial tissues was low at and after the mid-secretory phase of the menstrual cycle. Progesterone suppressed the gene expression of BMP7 in cultured ESC. Treatment with progesterone and estradiol for 12 days achieved decidualization of ESC, increasing the gene expression and protein secretion of IGFBP1. Addition of BMP7 protein to the culture almost completely inhibited these increases. BMP7 suppressed BrdU incorporation in ESC, which indicated an antiproliferative effect of BMP7 on ESC.

CONCLUSIONS: Progesterone-induced suppression of BMP7 and BMP7-induced inhibition of decidualization and proliferation of ESC suggest an elaborate regulatory mechanism for decidualization through BMP7 in the endometrium.

Key words: BMP7 / IGFBP1 / progesterone / decidualization / proliferation

Introduction

The endometrium undergoes dynamic changes during the menstrual cycle. Proper endometrial changes are essential for successful implantation, and aberrant endometrial status may lead to implantation failure. In addition to ovarian steroids, which have a central role in the regulation of morphological and functional changes to the endometrium, there are many local factors that modulate endometrial status (Kayisli *et al.*, 2004; Dimitriadis *et al.*, 2005).

Bone morphogenetic proteins (BMPs), together with growth differentiation factors (GDFs), comprise a subfamily of the transforming growth factor-β superfamily. BMPs and GDFs are multifunctional growth factors and their effects have been reported mainly in bone, cartilage, ligament and tendon formation (Francis-West *et al.*, 1999). However, BMPs and GDFs have also been demonstrated to control cellular proliferation, differentiation and apoptosis in reproductive tissues (Shimasaki *et al.*, 2004).

Gene expression of BMP2 (Ying and Zhao, 2000), BMP4 (Ying and Zhao, 2000), BMP6 (Lyons et al., 1989), BMP7 (Ozkaynak et al., 1997; Paria et al., 2001), GDF9 (Fitzpatrick et al., 1998) and GDF10 (Zhao et al., 1999) has been reported in the mouse uterus. These BMPs are expressed in a different spatiotemporal pattern and are thus speculated to have specific functions in the uterus. Mice deficient in ALK6, the receptor for these BMPs, have an abnormal endometrium and are infertile (Yi et al., 2001). A recent study has further demonstrated the presence of BMP2, BMP4, BMP7, GDF5, GDF8 and GDF11 in the human endometrium (Stoikos et al., 2008). BMP7 is unique among these BMPs in that its mRNA is lost from the uterine epithelium shortly after implantation in mice (Ozkaynak et al., 1997). In the human, gene expression of BMP7 has been reported in cultured endometrial stromal cells (ESC), with the expression level not being changed by cAMP-induced decidualization (Stoikos et al., 2008). In addition, immunostaining of human biopsied specimens have shown that BMP7 can be detected in highly decidualized cells with a vesicle staining pattern but not in first trimester decidua (Stoikos et al., 2008).

Although these findings imply a functional role for BMP7 in endometrial physiology, to date there have been no studies examining the effects of BMP7 on the endometrium. To determine the possible roles of BMP7 in the human endometrium, in the present study, we first examined the gene expression of BMP7 in the endometrium. We then studied the effects of BMP7 on decidualization of ESC, measuring insulin-like growth factor binding protein 1 (IGFBP1) as a marker of decidualization (Harada et al., 2006). We also examined the effects of BMP7 on proliferation of ESC.

Materials and Methods

Tissue samples

Endometrial tissue was obtained from 39 women, either by curettage under sterile conditions or from women undergoing hysterectomy for benign gynecologic disease. The mean (± SD) age of the women was 37.8 ± 8.2 years. All women had regular menstrual cycles and none had received hormonal treatment within the 6 months prior to surgery. The specimens were dated according to the women's menstrual history. In order to avoid contamination with trophoblast cells, decidual tissues were collected from five women with ectopic pregnancy but without uterine bleeding, by dilation and curettage according to previous studies (Koga et al., 2001; Hirota et al., 2005). The experimental procedures were approved by the institutional review board of the University of Tokyo, and all women provided written informed consent for the use of their endometrial tissue.

Isolation and culture of human ESC

ESC were isolated and cultured as described previously (Koga et al., 2001; Yoshino et al., 2003). Fresh endometrial biopsy specimens collected in sterile medium were rinsed to remove blood cells. Tissues were minced into small pieces and incubated in DMEM/F-12 containing type I collagenase (0.25%; Sigma, St Louis, MO, USA) and deoxynuclease I (15 U/ml; Takara, Tokyo, Japan) for 60 min at 37°C. The resulting dispersed endometrial cells were separated by filtration through a 40-μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA). Any intact endometrial epithelial glands that remained were retained by the strainer, whereas dispersed ESC passed through the strainer into the filtrate. ESC in the filtrate were collected by centrifugation at 250g and resuspended in phenol red-free DMEM/F-12 containing 5% charcoal-stripped fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B. The ESC were seeded in a 100-mm culture plate and kept at 37°C in a humidified atmosphere of 5%CO_2−95% air. At the first passage, cells were plated at a density of 1×10^5 cells/ml into 12- or 96-well culture plates (Becton Dickinson) and used for further treatments.

Treatment of ESC

To determine the effects of estrogen and progesterone on the gene expression of BMP7 in ESC, ESC were treated with 2.5% charcoal/dextran-treated (stripped) FBS (HyClone, Logan, UT, USA) in the presence of estradiol (10 ng/ml) or progesterone (100 ng/ml) for 6, 12 and 24 h. To examine the effect of BMP7 on decidualization, *in vitro* decidualization was achieved as described previously (Koga et al., 2001). Briefly, after cells had reached 70% confluence in 12-well culture plates, they were rinsed and treated with 2.5% charcoal/dextran-treated (stripped) FBS in the presence of estradiol (10 ng/ml) plus progesterone (100 ng/ml) or 0.1% ethanol vehicle (control) for 12 days. BMP7 (0, 10 or 100 ng/ml; R&D Systems, Minneapolis, MN, USA) was also added to the culture medium. Culture media were collected and replenished every 3 days.

Quantitative reverse transcription and real-time quantitative PCR

Total RNA was extracted from endometrial tissues and ESC using an RNeasy Mini Kit (Qiagen, Hilden, Germany). After reverse transcription, real-time quantitative PCR and data analysis were performed using a LightCycler (Roche Diagnostic, Mannheim, Germany), as reported previously (Harada et al., 2006). Expression of BMP7 and IGFBP1 mRNA was normalized for RNA loading for each sample using human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Toyobo) mRNA as an internal standard. The BMP7 primers chosen (sense: 5′-GCCTACTACTGTGAGGGGGAG -3′; antisense: 5′-GAAGTAGAGGACGGAGATGGC-3′) amplified a 163-bp fragment. The IGFBP1 primers chosen (sense: 5′-GAGAGCACGGAGATAACTGAGG-3′; antisense: 5′-TTGGTGACATGGAGAGCCTTCG-3′) amplified a 131-bp fragment. The PCR conditions were as follows: for BMP7, 40 cycles of: 95°C for 10 s, 64°C for 10 s and 72°C for 4 s; for IGFBP1, 40 cycles of: 95°C for 10 s, 67°C for 10 s and 72°C for 5 s; for GAPDH, 30 cycles of: 95°C for 10 s, 64°C for 10 s, 72°C for 18 s. All PCR conditions were followed by melting curve analysis.

Measurement of IGFBP-1 protein

Concentrations of IGFBP1 in the conditioned media were determined using a specific ELISA kit (R&D Systems, Minneapolis, MN, USA). The limit of sensitivity of the kit was 31.3 pg/ml. The concentrations measured were normalized against the total protein of cell lysates from each well of the culture plates.

5-Bromo-2′-deoxyuridine proliferation assay

The bromodeoxyuridine (BrdU) proliferation assay was performed as described previously (OuYang et al., 2008) using the Biotrak Cell Proliferation ELISA System (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Briefly, after incubation of ESC in serum-free medium for 24 h in 96-well plates, cells were treated for a further 24 h with serum-free medium containing either BMP7 (0, 10, 100 ng/ml) or 20% charcoal-stripped FBS as a positive control. After the 24 h incubation, 100 μl BrdU solution was added and cells were incubated at 37°C for an additional 2 h.

Statistically analysis

Expression of BMP7 mRNA in endometrial tissues was analyzed by the Kruskal–Wallis test, whereas other data were analyzed by ANOVA. Both tests were followed by *post hoc* analysis for multiple comparisons. $P < 0.05$ was considered significant.

Results

Expression of BMP7 mRNA in endometrial tissue throughout the menstrual cycle and in pregnancy and effect of progesterone and estradiol on ESC

As shown in Fig. 1, expression of BMP7 mRNA in endometrial tissues was significantly lower in the mid- and late secretory phases and in the decidua compared with expression in the mid-proliferative phase. In cultured ESC, treatment with progesterone, but not estradiol, decreased BMP7 mRNA expression at 12 and 24 h, compared with 0 h, in a time-dependent manner (Fig. 2A). Long-term culture of ESC in the presence of progesterone and estradiol remarkably decreased BMP7 mRNA expression on Day 3 and later, and distinctly induced IGFBP1 mRNA expression on Day 12 (Fig. 2B).

Effect of BMP7 on gene expression and secretion of IGFBP1

Treatment with estradiol and progesterone for 12 days induced IGFBP1 mRNA expression in ESC. However, the addition of 10 and 100 ng/ml BMP7 to the culture medium markedly decreased the expression of IGFBP1 mRNA induced by the hormonal treatment in ESC (Fig. 3A). Figure 3B shows secretion of IGFBP1 protein from ESC, which was induced by estradiol and progesterone treatment on Day 9 and was increased to higher levels on Day 12. The addition of BMP7 to the culture medium markedly reduced IGFBP1 protein secretion, to almost undetectable levels in the presence of 100 ng/ml BMP7.

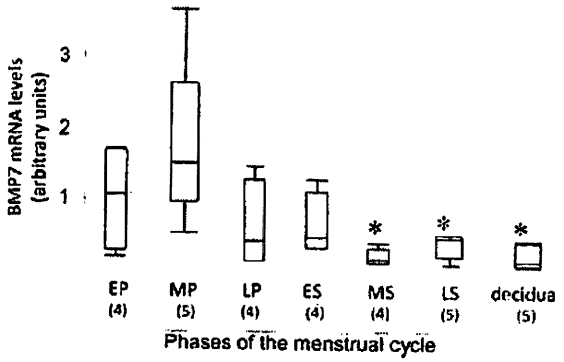

Figure 1 Expression of BMP7 mRNA in human endometrial tissues throughout the menstrual cycle and in early pregnant decidua.

Total RNA extracted from endometrial tissues and decidual tissues of ectopic pregnancies was reverse transcribed and then amplified by real-time PCR using primers for BMP7. Values were calculated by subtracting data for signal threshold cycles (Ct) of the internal standard (GAPDH) from Ct values for BMP7. The boxes represent the 25th and 75th percentiles. The median is denoted by the line that bisects the boxes. The whiskers indicate the extent of the data on the 1.5× interquartile range. *$P < 0.05$ compared with the MP. EP, early proliferative phase; MP, mid-proliferative phase; LP, late proliferative phase; ES, early secretory phase; MS, mid-secretory phase; LS, late secretory phase. The number of samples is shown in parentheses.

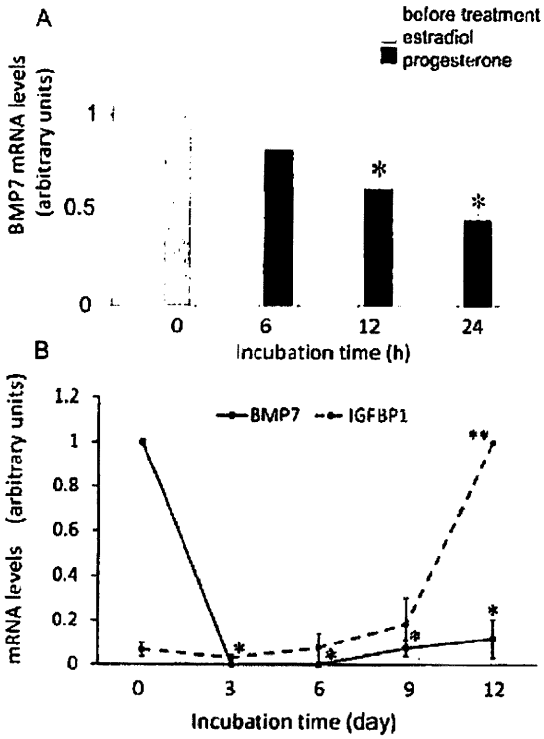

Figure 2 (**A**) Expression of BMP7 mRNA in ESC treated with estrogen (10 ng/ml) or progesterone (100 ng/ml) for 24 h. Data are the mean ± SEM of combined data from three independent experiments using different ESC from three patients. (**B**) Expression of BMP7 and IGFBP1 mRNA in ESC. *In vitro* decidualization of ESC was achieved by culturing ESC in the presence of estrogen (10 ng/ml) and progesterone (100 ng/ml) for 12 days. Data are the mean ± SEM of combined data from three independent experiments using different ESC from three patients. Total RNA isolated from ESC was reverse transcribed and then amplified by real-time PCR using primers for BMP7, IGFBP1 and GAPDH. Values were calculated by subtracting data for signal threshold cycles (Ct) of the internal standard (GAPDH) from Ct values for BMP7 or IGFBP1. (A) *$P < 0.05$ compared with 0 h. (B) *$P < 0.05$ compared with Day 0 (BMP7); **$P < 0.05$ compared with Day 0 (IGFBP1).

Effect of BMP7 on ESC proliferation

BMP7 at 10 and 100 ng/ml decreased BrdU incorporation in ESC by 20.5 ± 4.1 and 29.9 ± 4.2% (mean ± SEM of six replicate cultures) of the untreated controls, respectively (both $P < 0.05$ compared with the control), although 20% charcoal-stripped FBS increased BrdU incorporation by 134.8 ± 11.2%.

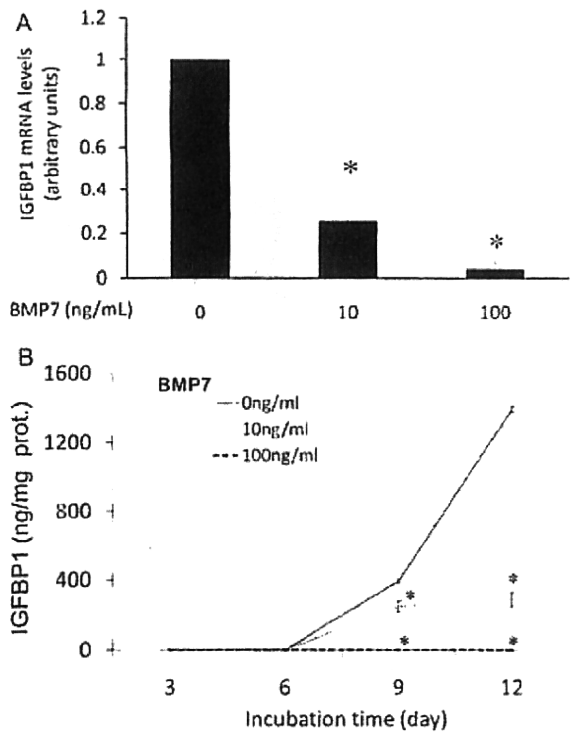

Figure 3 Effects of BMP7 on gene expression and protein secretion of IGFBP1 from ESC. (**A**) Effects of 10 and 100 ng/ml BMP7 on IGFBP1 mRNA expression in ESC treated with a combination of 10 ng/ml estradiol plus 100 ng/ml progesterone (EP) for 12 days. Total RNA isolated from ESC was reverse transcribed and then amplified by real-time PCR using primers for IGFBP1. Values were calculated by subtracting data for signal threshold cycles (Ct) of the internal standard (GAPDH) from Ct values for IGFBP1. Values are the mean ± SEM of four independent experiments using samples from four different patients. $^*P < 0.05$ versus 0 ng/ml. (**B**) IGFBP1 concentrations in culture media of ESC treated with EP, with or without BMP7 (10 and 100 ng/ml), for 3, 6, 9 and 12 days. IGFBP1 concentrations were determined using a specific ELISA and normalized against the total protein of cell lysates from each well. Data are the mean ± SEM of duplicate cultures. $^*P < 0.05$ compared with the respective control on each day. The result is representative of three separate experiments using samples from three different patients.

Discussion

In the present study, we demonstrated that gene expression of BMP7 in the endometrium was lower in the mid- and late secretory phases and in early pregnancy than in the mid-proliferative phase. Progesterone, but not estradiol, decreased BMP7 gene expression in ESC, which was significant after 12 h. Long-term incubation with progesterone and estradiol induced IGFBP1 protein secretion from ESC, which was inhibited by BMP7. BMP7 also decreased ESC proliferation.

In parallel with dynamic changes in the endometrium, the expression of many molecules in the endometrium changes spatiotemporally. Because embryos are accepted by the endometrium only during the 'implantation window', which corresponds to the mid-secretory phase, those substances for which levels in the endometrium change during the mid-secretory phase may have a role in preparing the receptive endometrium. In this context, the decrease in the gene expression of BMP7 in the mid-secretory phase may contribute to the development of the receptive endometrium.

Decidualization is a process in which remarkable structural and functional changes occur in ESC to prepare an appropriate environment for embryo implantation and maintenance of pregnancy. Decidualization is regulated by the ovarian steroid hormones estradiol and progesterone. In addition, the importance of other factors in the induction of decidualization has been demonstrated recently. For example, we found that mechanical stretch augments decidualization (Harada *et al.*, 2006), and others have found that paracrine factors are involved in decidualization (Tang *et al.*, 1994; Fazleabas and Strakova, 2002). The results of the present study, showing that BMP7 suppresses secretion of IGFBP1 protein from decidualizing ESC, suggest that BMP7 may act as an antidecidualization factor in the endometrium.

The antidecidualization activity of BMP7 is in marked contrast with the actions of BMP2, which increases the secretion of IGFBP1 and prolactin, another marker of decidualization, in decidualized ESC (Li *et al.*, 2007; Stoikos *et al.*, 2008). The expression patterns of BMP2 and BMP7 in the endometrium also appear to be different because *in-vitro* decidualization increases the expression of BMP2 in ESC (Li *et al.*, 2007). Thus, as a result of their different spatiotemporal expression, it is possible that the opposing actions of these two BMPs support decidualization and the subsequent establishment of pregnancy. From a therapeutic perspective, therapies targeted for BMP7 and BMP2 could be applicable for the treatment of implantation failure caused by impaired decidualization. Interestingly, the opposing functions of BMP7 and BMP2 have been demonstrated recently in adipogenesis, with BMP7 contributing to the development of brown adipocytes and BMP2 contributing to the development of white adipocytes (Tseng *et al.*, 2008).

The decrease in BMP7 expression in the decidualized endometrium may also be important for the successful development of the placenta. It has been shown that BMP7 suppresses the production of human chorionic gonadotrophin and progesterone from the trophoblast (Martinovic *et al.*, 1996). Because these hormones are tremendously important for the maintenance of pregnancy, the presence of BMP7 in the endometrium would be problematic for invading trophoblasts. Therefore, reduced BMP7 expression may be necessary not only for the development of a receptive endometrium, but also for the invading trophoblasts to establish pregnancy.

Progesterone inhibited BMP7 gene expression in ESC. This suggests that the decreased expression of BMP7 in the endometrium from the mid-secretory phase is due to the effects of progesterone. Notably, the inhibition of BMP7 gene expression by progesterone was clearly observed as early as 12 h. In addition, the decrease in BMP7 expression evidently preceded the increase in IGFBP1 expression during decidualization with progesterone and estradiol. This result, however, appears to be inconsistent with the findings by Stoikos *et al.* (2008) which showed that BMP7 gene expression was not altered by *in vitro* decidualization with cAMP. This difference may indicate that progesterone is prerequisite for down-regulation of BMP7 expression in the process of decidualization. Collectively,

progesterone may suppress BMP7 gene expression in the early stage to facilitate subsequent decidualization. Another apparently inconsistent finding of Stoikos et *al.* (2008) was the vesicular staining for BMP7 in decidual cells in mid–late secretory endometrium although staining patterns were not shown in other phases of the menstrual cycle. The decrease in BMP7 gene expression by progesterone might be involved in the change, if any, of intracellular localization of BMP7. Another possible explanation for the inconsistency may be any cross-reactivity of the antibody used in that study.

BMP7 appears to stimulate or inhibit proliferation depending on the cell type; for example, BMP7 stimulates proliferation of ovarian granulosa cells (Lee et *al.*, 2001) and Sertoli cells (Puglisi et *al.*, 2004), but inhibits proliferation of aortic smooth muscle cells (Doral et *al.*, 2000), renal mesangial cells (Otani et *al.*, 2007) and prostate cancer cells (Miyazaki et *al.*, 2004). In the present study, BMP7 inhibited the proliferation of ESC. Thus, the decrease in BMP7 expression in the decidualized endometrium may contribute to the proliferation of decidual cells during pregnancy.

The present study has some limitations. First, the decidual tissues of ectopic pregnancies used in this study have advantages in that they are free from contamination with trophoblast cells, but they may have different characteristics from deciduas of normal pregnancies. Second, we measured mRNA levels but not protein levels of BMP7. Although cellular protein levels shown by immunostaining or immunoblotting are not necessarily proportional to their functional activities, knowledge about them would help our understanding of BMP7 in the endometrium. A further study is warranted regarding this point.

In summary, the results of the present study suggest that progesterone decreases BMP7 expression in the endometrium. The decrease in BMP7 expression may facilitate decidulalization of the endometrium, thus aiding the establishment of pregnancy.

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Involvement of a novel preimplantation-specific gene encoding the high mobility group box protein *Hmgpi* in early embryonic development

Mitsutoshi Yamada[1,2], Toshio Hamatani[1,*], Hidenori Akutsu[2], Nana Chikazawa[1,2], Naoaki Kuji[1], Yasunori Yoshimura[1] and Akihiro Umezawa[2]

[1]Department of Obstetrics and Gynecology, Keio University School of Medicine, 35 Shinanomachi Shinjyuku-ku, Tokyo 160-8582, Japan and [2]Department of Reproductive Biology, National Research Institute for Child Health and Development, 2-10-1 Ohkura Setagaya-ku, Tokyo 157-8535, Japan

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Mining gene-expression-profiling data identified a novel gene that is specifically expressed in preimplantation embryos. *Hmgpi*, a putative chromosomal protein with two high-mobility-group boxes, is zygotically transcribed during zygotic genome activation, but is not transcribed postimplantation. The *Hmgpi*-encoded protein (HMGPI), first detected at the 4-cell stage, remains highly expressed in pre-implantation embryos. Interestingly, HMGPI is expressed in both the inner cell mass (ICM) and the trophectoderm, and translocated from cytoplasm to nuclei at the blastocyst stage, indicating differential spatial requirements before and after the blastocyst stage. siRNA (siHmgpi)-induced reduction of *Hmgpi* transcript levels caused developmental loss of preimplantation embryos and implantation failures. Furthermore, reduction of *Hmgpi* prevented blastocyst outgrowth leading to generation of embryonic stem cells. The siHmgpi-injected embryos also lost ICM and trophectoderm integrity, demarcated by reduced expressions of Oct4, Nanog and Cdx2. The findings implicated an important role for *Hmgpi* at the earliest stages of mammalian embryonic development.

INTRODUCTION

Preimplantation development encompasses the period from fertilization to implantation. Oocytes cease developing at metaphase of the second meiotic division, when transcription stops and translation is reduced. After fertilization, sperm chromatin is reprogrammed into a functional pronucleus and zygotic genome activation (ZGA) begins, whereby the maternal genetic program governed by maternally stored RNAs and proteins must be switched to the embryonic genetic program governed by *de novo* transcription (1,2). Our previous gene expression profiling during preimplantation development revealed distinctive patterns of maternal RNA degradation and embryonic gene activation, including two major transient 'waves of *de novo* transcription' (3). The first wave during the 1- to 2-cell stage corresponds to ZGA. The second wave during the 4- to 8-cell stage, known as mid-preimplantation gene activation (MGA), induces dramatic morphological changes to the zygote including compaction and blastocele formation, particularly given that few genes show large expression changes after the 8-cell stage. ZGA and MGA together generate a novel gene expression profile that delineates the totipotent state of each blastomere at the cleavage stage of embryogenesis, and these steps are prerequisite for future cell lineage commitments and differentiation. The first such differentiation gives rise to the inner cell mass (ICM), from which embryonic stem (ES) cells are derived, as well as the trophectoderm at the blastocyst stage. However, the molecular regulatory mechanisms underlying this preimplantation development and ES-cell generation from the ICM remain unclear.

Induced pluripotent stem (iPS) cells are ES cell-like pluripotent cells, generated by the forced expression of defined factors in somatic cells, including Pou5f1/Oct4, Sox2, Klf4

*To whom correspondence should be addressed. Tel: +81 353633819; Fax: +81 332261667; Email: t-hama@sc.itc.keio.ac.jp

and Myc (4). These iPS factors are thought to reprogram somatic nuclei in a somewhat similar way as ooplasm does in reconstructed oocytes by nuclear transfer (NT). However, with the exception of Oct4, these factors are not highly expressed maternally in oocytes, and only increased by zygotic transcription during preimplantation, based on expression sequence tag (EST) frequencies in Unigene cDNA libraries and microarray data from oogenesis to preimplantation development (5). Although pluripotency is achieved within 2 days in NT embryos reconstructed with a somatic nucleus, it takes approximately 2 weeks for the establishment of iPS cells. Such immediate induction of pluripotency during preimplantation development is attributed to well-organized transcriptional regulation, i.e. waves of transcription whereby maternal gene products trigger ZGA, which in turn fuels MGA. On the other hand, the forced simultaneous transcription of iPS factors in somatic cells does not efficiently induce these waves of transcription, and it takes a long time to activate the other genes necessary for pluripotency. Studying transcriptional regulation during preimplantation development would therefore also help unravel the establishment of iPS cells as well as pluripotency in these cells.

Large-scale EST projects (6–8) and DNA microarray studies (3,9–11) have revealed many novel genes zygotically expressed during preimplantation development. Very few of these genes, however, are exclusively expressed in preimplantation embryos (12), and such genes ought to have important roles during preimplantation development. For example, *Zscan4*, a novel transcription factor, is expressed specifically in 2-cell stage embryos and a subset of ES cells (13). Reduction of *Zscan4* transcript levels by siRNAs delays progression from the 2-cell to the 4-cell stage, and produces blastocysts that neither implant *in vivo* nor proliferate in blastocyst outgrowth culture. Thus, a transcription factor expressed exclusively in preimplantation embryos is potentially a key regulator of global gene expression changes during preimplantation development. On the other hand, reprogramming gene expression during ZGA and MGA requires considerable changes in chromatin structure (14–16), and modulation of chromatin folding affects access of regulatory factors to their cognate DNA-binding sites. This modulation can be achieved by loosening the chromatin structure, by disrupting the nucleosome structure, by DNA bending and unwinding, and by affecting the strength of DNA-histone interactions via postsynthetic modifications of histones (17,18). Many of these structural changes are mediated by a large and diverse superfamily of high-mobility-group (HMG) proteins, which are the second most abundant chromosomal proteins after histones (18).

This study identified a novel preimplantation-specific gene, *Hmgpi*, which encodes a chromosomal protein containing HMG box domains. It reports a detailed expression analysis of *Hmgpi* and the *Hmgpi*-encoded protein (HMGPI), which was translocated from the cytoplasm to nuclei at the blastocyst stage. Loss-of-function studies were also conducted using siRNA technology. The siRNA-induced reduction in *Hmgpi* expression caused developmental loss at preimplantation stages and hampered implantation through reduced proliferation of both ICM-derived cells and trophectodermal cells during peri-implantation development.

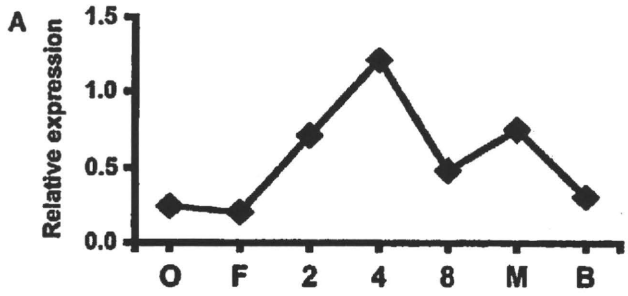
RESULTS

Gene structure of a preimplantation-stage-specific gene, *Hmgpi*

In silico analysis identified *Hmgpi* (an **HMG**-box protein, preimplantation-embryo-specific) as a preimplantation-stage-specific gene encoding a chromosomal protein containing HMG box domains. The *Hmgpi* transcript levels are probably upregulated during ZGA (1- to 2-cell stages) to peak at the 4-cell stage, based on gene expression profiling (3,9) (Fig. 1A). Using the public expressed-sequence tag (EST) database, 16 cDNA clones were found exclusively in preimplantation-embryo libraries (2- to 8-cell stages) (Fig. 1B). One of these contained the full *Hmgpi* gene coding sequence (AK163257) (Fig. 1C), spanning 2579 bp and split into two exons, which encode a protein of 394 amino acids (aa) (NP_001028965) harboring a SANT domain ('SWI3, ADA2, N-CoR, and TFIIIB' DNA-binding domain) and two HMG-box domains, based on SMART domain prediction analysis (19) (Fig. 1C). In the NCBI Gene database, the *Hmgpi* gene is called Ubtf-like 1 (*Ubtfl1*) based on aa sequence similarity (36% identity and 58% positives by BLAST) to *Ubtf*-encoded protein 'upstream binding transcription factor', which contains a SANT domain and six HMG-box domains. Two rat homologs (*Ubtfl1* and *RGD1304745*) and three human homologs (*UBTFL1-3*) of the mouse *Hmgpi* were identified by BLASTing of NP_001028965 against the NCBI nucleotide database. Pairwise alignment scores by BLAST of amino acid sequences for rat and human homologs are 72.3–72.5% and 53.8–54.1%, respectively (Fig. 1D and Supplementary Material, Table S1), while those for nucleotide sequences are 83.7 and 66.8–67.0%, respectively. All these human homologs were predicted by *in silico* genome-based analysis, and have no ESTs in the Unigene database. The absence of human ESTs may reflect the paucity of cDNA libraries of human preimplantation embryos in the Unigene database, despite specific expression of the Hmgpi gene in human preimplantation embryos. Based on the number and the type of HMG-box domains, this novel protein could also be categorized into the HMG-box family (HMGB). A dendrogram of aa sequence similarity in HMG family proteins indicates two HMG subgroups (Fig. 1E). One includes the HMG-nucleosome binding family (HMGN) and the HMG-AT-hook family (HMGA), and the other is HMGB that includes HMGPI. All members of HMGB contain two HMG-box domains ('HMG-box' or 'HMG-UBF_HMG-box').

Expression of the *Hmgpi* gene and protein

First, we experimentally confirmed the preimplantaion-stage-specific expression pattern of *Hmgpi* suggested by the *in silico* analysis. Northern blot analysis using a mouse multiple tissue poly(A)RNA panel (FirstChoice® Mouse Blot 1 from Ambion, Austin, TX, USA) failed to detect expression of the *Hmgpi* gene (data not shown). While RT–PCR analysis using cDNA isolated from mouse adult tissues and fetuses (E7, E11, E15 and E17) also failed to show *Hmgpi* expression, RT–PCR analysis for preimplantation embryos indicated *Hmgpi* expression from the 2-cell embryo to the blastocyst



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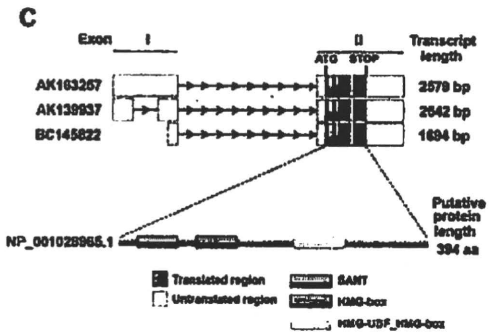
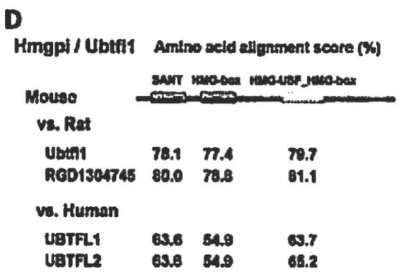
Developmental stage	Gene EST	Total EST in pool
Oocyte	0	19488
Unfertilized ovum	0	20366
Zygote	0	26382
Cleavage	16	65852
Blastocyst	0	69936
Egg cylinder	0	12257
Gastrula	0	28673
Organogenesis	0	128591
Fetus	0	670244
Neonate	0	107177
Juvenile	0	293396
Adult	0	1026072





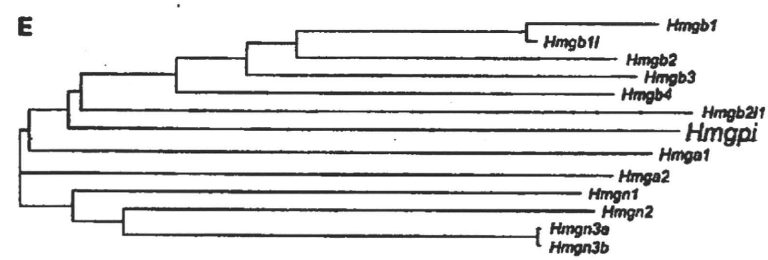


Figure 1. *In silico* analysis of *Hmgpi* expression. (A) Previous microarray analysis of *Hmgpi* expression. *Hmgpi* expression appeared at the 2-cell stage, peaked at the 4-cell stage and then decreased (3). (B) Expression sequence tag (EST) frequencies in Unigene cDNA libraries. Out of 4.7 million mouse ESTs, 16 *Hmgpi* clones were exclusively detected at the cleavage stages: 9, 2 and 5 ESTs from 2-cell, 4-cell and 8-cell libraries, respectively. (C) Exon–intron structures and a putative protein structure of *Hmgpi*. *Hmgpi* has three exon–intron models and one protein model. Predicted protein domains are also shown. (D) Conserved domains of *Hmgpi*/*Ubtfl1* gene in mouse, rat and human. Pairwise alignment scores of conserved domains between species were shown. (E) Phylogenetic tree of gene nucleotide acid sequences containing HMG domains determined by a sequence distance method and the neighbour-joining (NJ) algorithm (41) using Vector NTI software (Invitrogen, Carlsbad, CA, USA).

stage (Fig. 2A). Furthermore, significant expression of *Hmgpi* was detected in ES cells, although not in embryonic carcinoma (EC) cells nor in mesenchymal stem cells (Fig. 2B). The relative abundance of *Hmgpi* transcripts in preimplantation embryos was measured by real-time quantitative RT–PCR (qRT–PCR) analysis (Fig. 2C). Four independent experiments were conducted with four replicates of 10 embryos each. To normalize the qRT–PCR reaction efficiency, *H2afz* was used as an internal standard (20). *Hmgpi* mRNA levels increased during the 1- to 2-cell stage, peaked at the 4-cell stage, and then gradually decreased during the 8-cell to blastocyst stage (Fig. 2C). The *in silico*-predicted preimplantation-stage-specific expression pattern of *Hmgpi* was therefore validated.

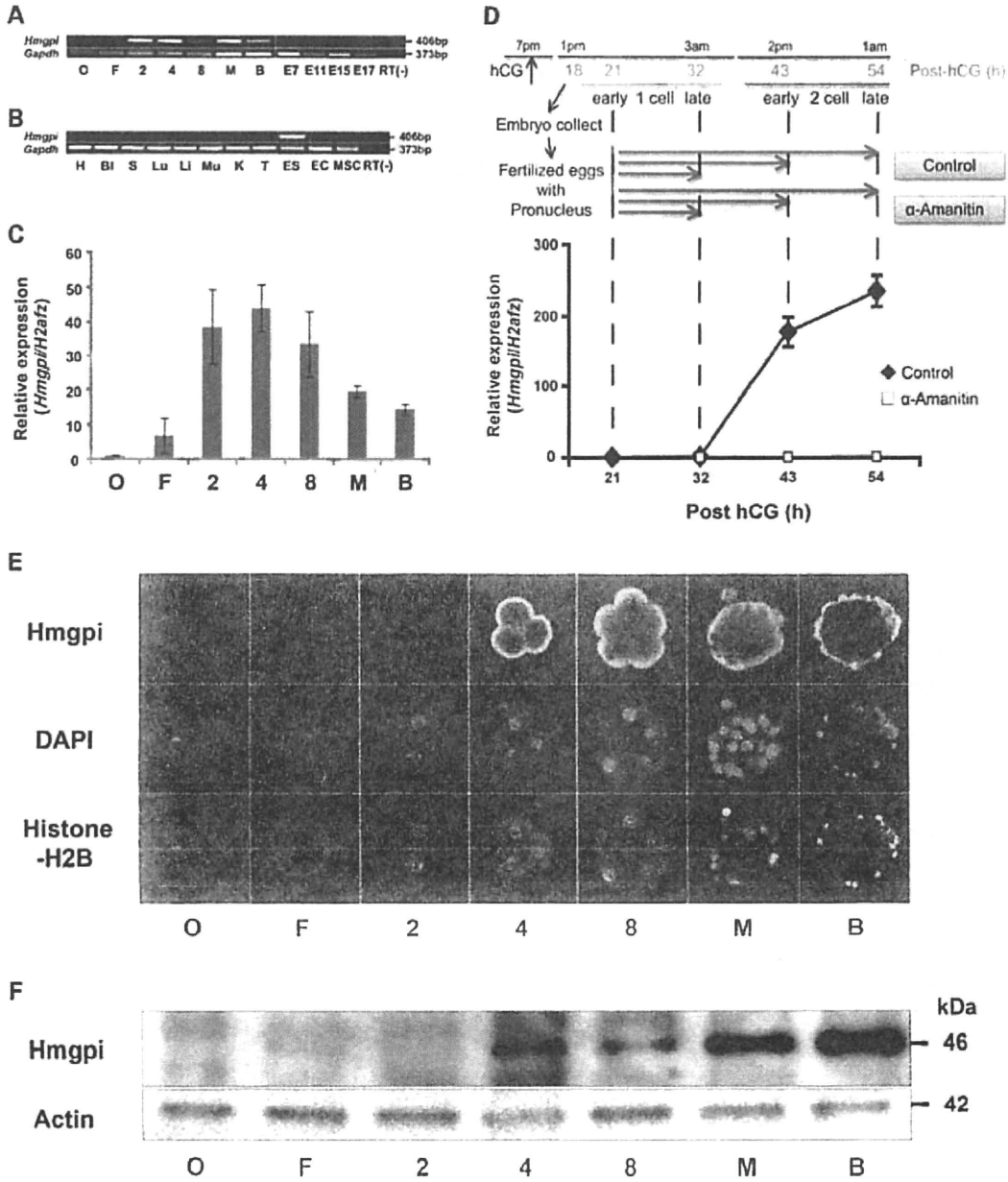


Figure 2. Expression of *Hmgpi* in preimplantation embryos and other tissues. (**A**) RT–PCR analysis of *Hmgpi* expression during preimplantation and postimplantation development (E7–E17). Three sets of 10 pooled embryos were collected from each stage (O: oocyte, F: fertilized egg, 2: 2-cell embryo, 4: 4-cell embryo, 8: 8-cell embryo, M: morula, and B: blastocyst) and used for RT–PCR analysis. The predicted sizes of the PCR products of *Hmgpi* and *Gapdh* are 406 and 373 bp, respectively. No PCR products were detected in the no-RT negative control (4-cell embryo). (**B**) RT–PCR analysis of *Hmgpi* expression in adult tissues, ES cells, EC cells and mesenchymal stem cells. mRNA was isolated from mouse tissues (H: heart, Bl: bladder, S: spleen, Lu: lung, Li: liver, Mu: muscle, K: kidney, T: testis, ES: ES cells, EC: EC cells, and MSC: mesenchymal stem cells). No PCR products were detected in the no-RT negative control (ES cells). (**C**) Real-time quantitative RT–PCR analysis of *Hmgpi* expression during preimplantation development. Fold differences in amounts of *Hmgpi* mRNA from the same numbers of oocytes (O), fertilized eggs (F), 2-cell embryos (2), 4-cell embryos (4), 8-cell embryos (8), morulae (M) and blastocysts (B) are shown after normalization to an internal reference gene (mouse *H2afz*). Values are means ± SE from four separate experiments. (**D**) *De novo* (zygotic) transcription of the *Hmgpi* gene. α-Amanitin studies revealed that *Hmgpi* is transcribed zygotically, but not maternally. *Hmgpi* expression was not observed before the 2-cell stage and α-amanitin completely inhibited *de novo* transcription at the 2-cell stage (closed rhombus: control group, open square: α-amanitin-treated group). The expression levels were normalized using *H2afz* as a reference gene. Values are means ± SE from four separate experiments. (**E**) Immunocytochemical analysis of HMGPI expression. MII oocytes and preimplantation embryos were immunostained with an anti-HMGPI antibody (red) and an anti-Histone-H2B antibody as a positive control of nuclear staining (green). Nuclei are shown by DAPI staining (blue). HMGPI protein was detected from 4-cell embryos to blastocysts. (**F**) Immunoblot analysis of HMGPI during preimplantation development. An amount of extracted protein corresponding to 100 oocytes or embryos was loaded per lane. Actin was used as a loading control. The representative result is shown from three independent experiments.

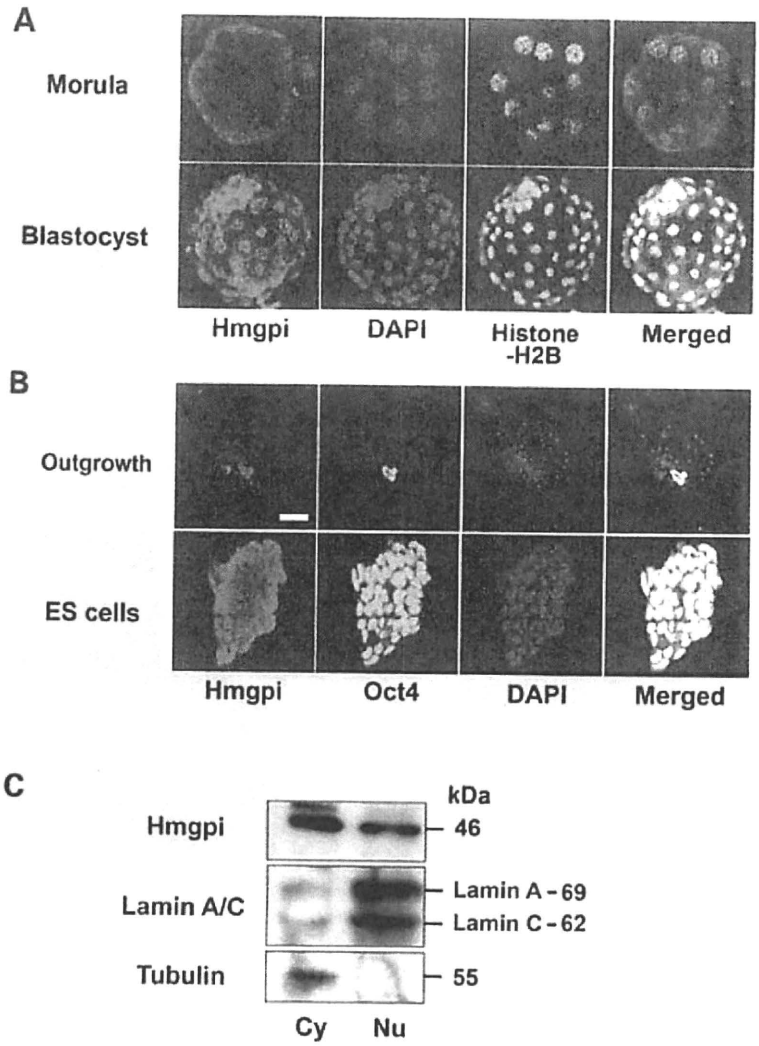


Figure 3. Localization of HMGPI in preimplantation embryos. (**A**) Nuclear translocation of HMGPI protein at the blastocyst stage. HMGPI was mainly detected in the cytoplasm of preimplantation embryos (from 4-cell embryos to morulae), but in the nuclei of blastocysts. Nuclei are shown by immunostaining with an anti-Histone-H2B antibody (green) and DAPI staining (blue). (**B**) Confocal microscopy images of blastocyst outgrowth and ES cells stained with antibodies to *Hmgpi* and *Oct4*, and with DAPI. Scale bar = 50 μM. (**C**) Western blotting analysis of HMGPI in cytoplasmic (Cy) and nuclear (Nu) fractions of ES cells. Lamin A/C and tubulin were used as markers of the nuclear and cytoplasmic fractions, respectively.

We then performed qRT–PCR analysis using α-amanitin to investigate *de novo* (zygotic) transcription of the *Hmgpi* gene. The supplementation of α-amanitin during *in vitro* culture from the 1-cell stage significantly reduced *Hmgpi* mRNA expression in the 2-cell embryos at post-hCG 43 and 53 h (early and late 2-cell stage, respectively) (Fig. 2D), implying that *Hmgpi* is transcribed zygotically during the major burst of ZGA, but not maternally.

To study the temporal and spatial expression pattern of the *Hmgpi*-encoded protein (HMGPI), we raised a polyclonal antibody against *Hmgpi* peptides. Western blot analysis of extracts from the mouse blastocysts showed only a single band corresponding to 46 kDa detected by the anti-HMGPI antibody. In addition, preincubation with the HMGPI peptide antigen abolished detection of the HMGPI protein, while preincubation with a control peptide had no effect on the immunodetection (Supplementary Material, Fig. S1). Although *Hmgpi* transcription started at the 2-cell stage, peaked at the 4-cell stage and then gradually decreased until the blastocyst stage (Fig. 2C), immunostaining and immunoblotting analysis revealed HMGPI expression from the 4-cell stage until the blastocyst stage, indicating a delayed expression pattern of HMGPI compared with that of the *Hmgpi* transcript. It was also notable that both ICM cells and trophectodermal cells retained HMGPI expression in blastocysts.

On the other hand, immunostaining for HMGPI in preimplantation embryos showed a unique subcellular localization pattern. Although a putative nuclear protein due to its role

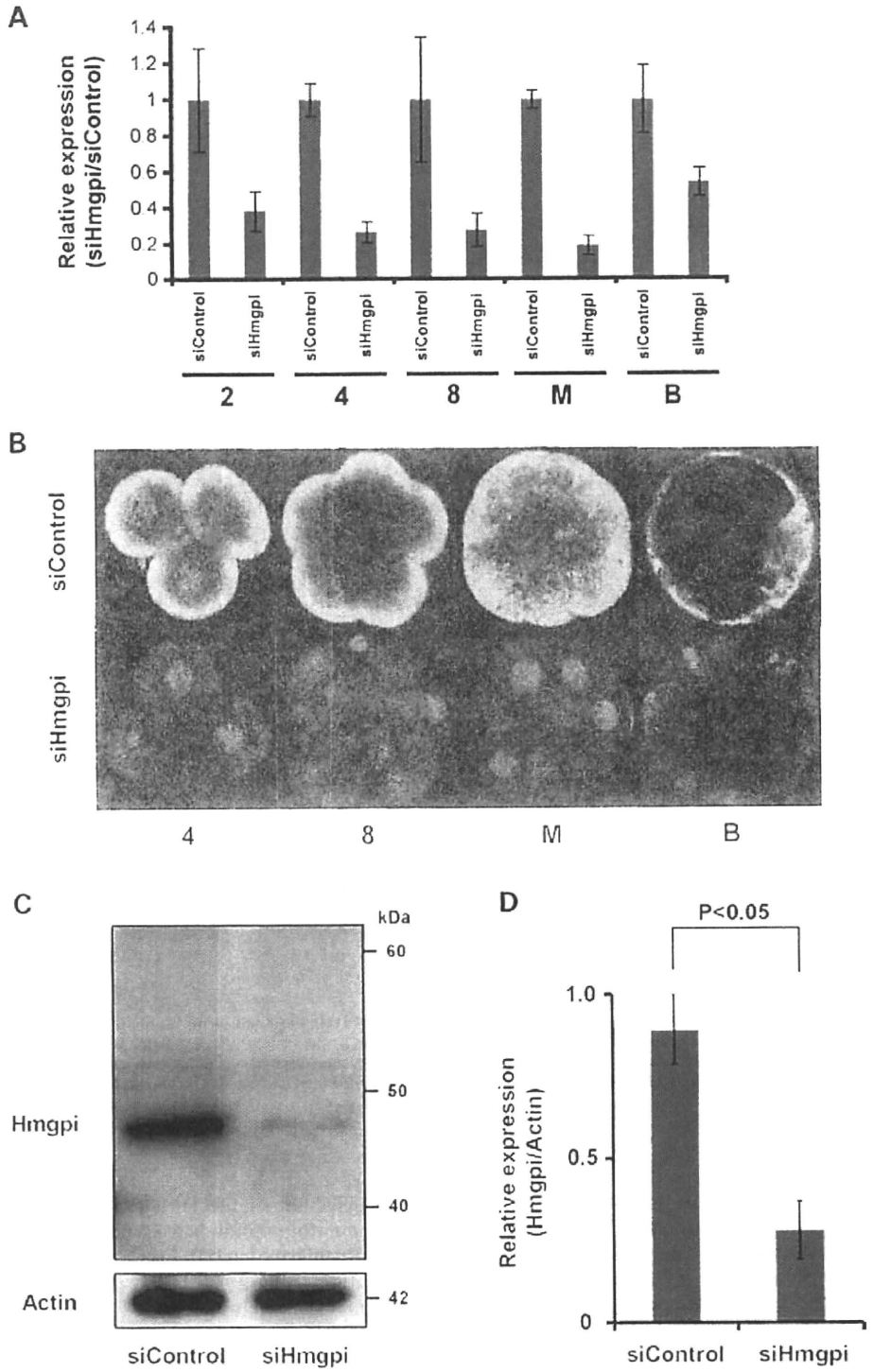


Figure 4. Loss-of-function study by siRNA technology. (**A**) Transcript levels of *Hmgpi* in embryos injected with control siRNA (siControl) and *Hmgpi* siRNA (siHmgpi) by real-time quantitative RT–PCR analysis. The expression levels were normalized using *H2afz* as a reference gene. Values are means ± SE for four separate experiments. (**B**) Laser scanning confocal microscopy images of HMGPI protein expression in a 4-cell embryo, 8-cell embryo, morula and blastocyst after injection with siControl or siHmgpi (red, HMGPI; blue, chromatin). (**C** and **D**) Immunoblot analysis of HMGPI expression at the blastocyst stage in siControl-injected and siHmgpi-injected embryos. The relative amount of HMGPI (46 kDa) was determined at the blastocyst stage (left: siControl-injected embryos, right: siHmgpi-injected embryos). The expression levels were normalized using actin expression (42 kDa) as a reference. Values are means ± SE from three separate experiments.

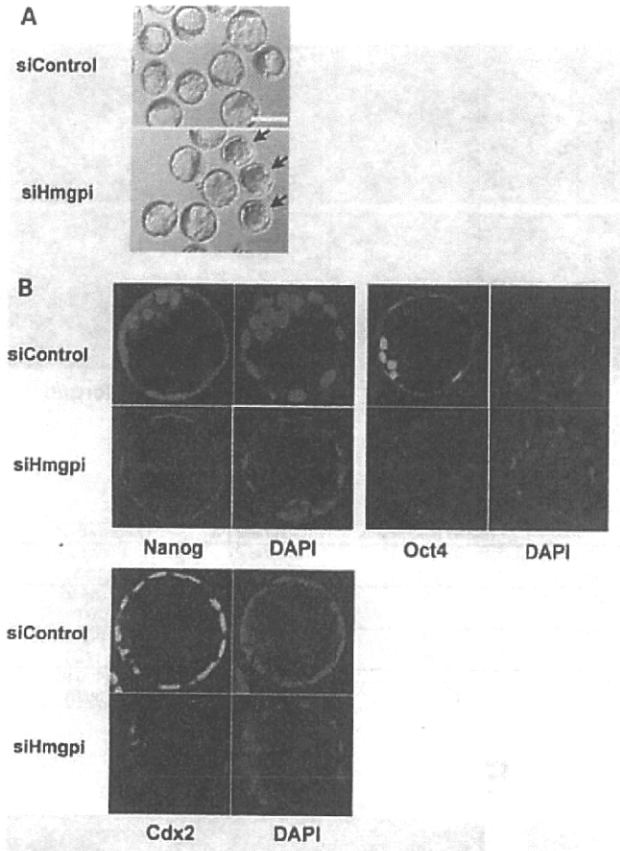


Figure 5. Function of *Hmgpi* in preimplantation development. (A) A pair of representative photos showing the development of embryos injected with *Hmgpi* siRNA (siHmgpi) and Control siRNA (siControl). The siHmgpi-injected embryos arrested at the morula stage are indicated by arrows. Scale bar = 100 μM. (B) For Nanog, Oct4 and Cdx2 immunostaining, all blastocysts in the siHmgpi-injected and siControl-injected groups were processed simultaneously. The laser power was adjusted so that the signal intensity was below saturation for the developmental stage that displayed the highest intensity and all subsequent images were scanned at that laser power. This allowed us to compare signal intensities for Nanog, Oct4 and Cdx2 expression between the siHmgpi-injected and siControl-injected embryos (Supplementary Material, Table S2).

as a transcription factor, HMGPI was detected mainly in the cytoplasm without any evidence of a nuclear localization from the 4-cell to the morula stage, suggesting a role other than transcriptional regulation (Fig. 2E). In contrast, HMGPI was localized to the nuclei rather than to the cytoplasm of blastocysts (Figs 2E and 3A). During blastocyst outgrowth, HMGPI was expressed in the nuclear region of most outgrowing cells, with scant amounts detected in the cytoplasm (Fig. 3B). Interestingly, Oct4-positive cells derived from the ICM showed particularly strong positive staining for HMGPI in the nucleus, suggesting a specific role as a nuclear protein in ES cells (Fig. 3B). On more closely examining HMGPI in ES cells, we found that almost all the Oct4-positive undifferentiated ES cells in a colony also expressed HMGPI (Fig. 3B), and immunoblotting confirmed HMGPI expression in both nuclear and cytoplasmic fractions of ES cells (Fig. 3C).

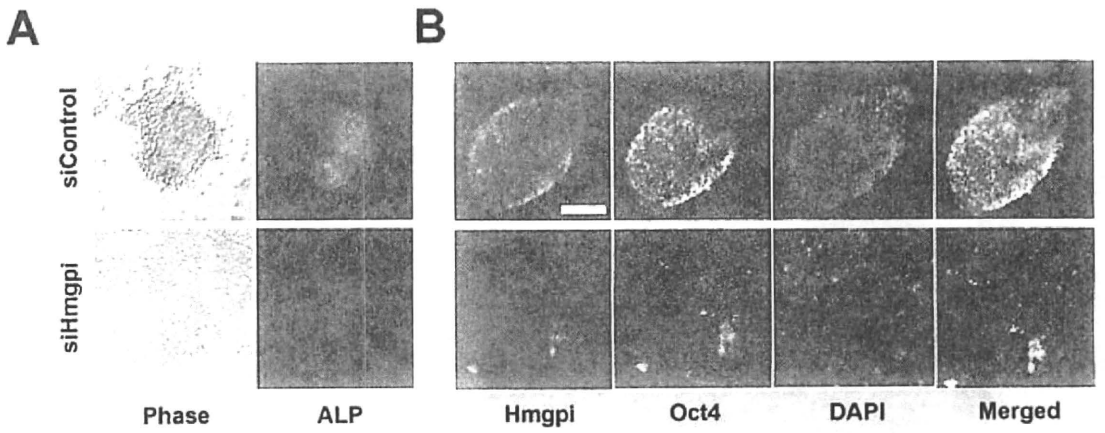
Effect of siRNA on *Hmgpi* mRNA level and protein synthesis

To investigate a role of *Hmgpi* in early embryonic development, we knocked down *Hmgpi* expression in mouse preimplantation embryos. We employed an oligonucleotide-based siRNA (denoted here siHmgpi and obtained from PE Applied Biosystems, Foster City, CA, USA). Zygotes injected with *Hmgpi* siRNAs (siHmgpi) or control siRNA (siControl) and non-injected zygotes as negative controls were cultured. *Hmgpi* expression was severely suppressed in the siHmgpi-injected embryos, and significantly lower than those in the siControl-injected or non-injected embryos (Fig. 4A). The siControl-injected embryos did not show any difference from the non-injected embryos in *Hmgpi* expression (data not shown). In addition, immunofluorescent staining clearly demonstrated that the siRNA injection reduced HMGPI protein expression in an individual preimplantation embryo (Fig. 4B). In the same set of experiments, the HMGPI levels were also assayed by western blotting (Figs 4C and 4D). HMGPI expression was significantly reduced in siHmgpi-injected blastocysts (0.89 ± 0.10) compared with that in negative controls (0.28 ± 0.08; $P < 0.05$).

Furthermore, we confirmed that siHmgpi had no influence on the expression of other genes with sequence similarities to *Hmgpi*, namely *Ubtf*, *Hmgb1*, *Hmgb2* and *Hmgb3*. Although *Ubtf*, *Hmgb1*, *Hmgb2* and *Hmgb3* were all expressed in control preimplantation embryos, the siHmgpi construct used in this study did not affect the expression of these genes in the siHmgpi-injected embryos (Supplementary Material, Fig. S2). On the other hand, it has been demonstrated that loss-of-function of these genes produces no distinct phenotypes at the pre- and peri-implantation stages (21).

Effect of *Hmgpi* siRNA on preimplantation development

To study the function of *Hmgpi* during preimplantation development, siHmgpi-injected or siControl-injected zygotes were cultured *in vitro* until the blastocyst stage. The embryos injected with siHmgpi at 21–23 h after hCG administration often failed to become blastocysts at 3.5 days postcoitum (dpc) (Fig. 5A). In addition, the reduction in Hmgpi expression significantly suppressed preimplantation development, whereby $68.9 \pm 1.3\%$ of siHmgpi-injected embryos became blastocysts, while $94.1 \pm 1.3\%$ of siControl-injected embryos reached the blastocyst stage (Supplementary Material, Fig. S3; $P < 0.0001$). Most of the siHmgpi-injected embryos that failed to become blastocysts showed developmental arrest after the morula stage and did not appear to form blastocoels, suggesting impairment of trophectodermal development (Supplementary Material, Fig. S3). To analyze the phenotype of siHmgpi-injected embryos further, we performed immunofluorescence staining of lineage-specific markers such as Cdx2, Nanog and Oct4 at the blastocyst stage. Although siHmgpi-injected embryos that reached the blastocyst stage appeared morphologically intact, the expression of lineage-specific markers was reduced (Fig. 5B). Cdx2, which is required for implantation and extraembryonic development, was particularly and markedly downregulated in trophectodermal cells, while Nanog and Oct4



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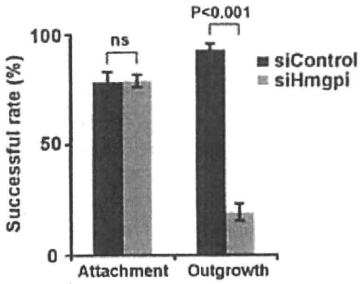
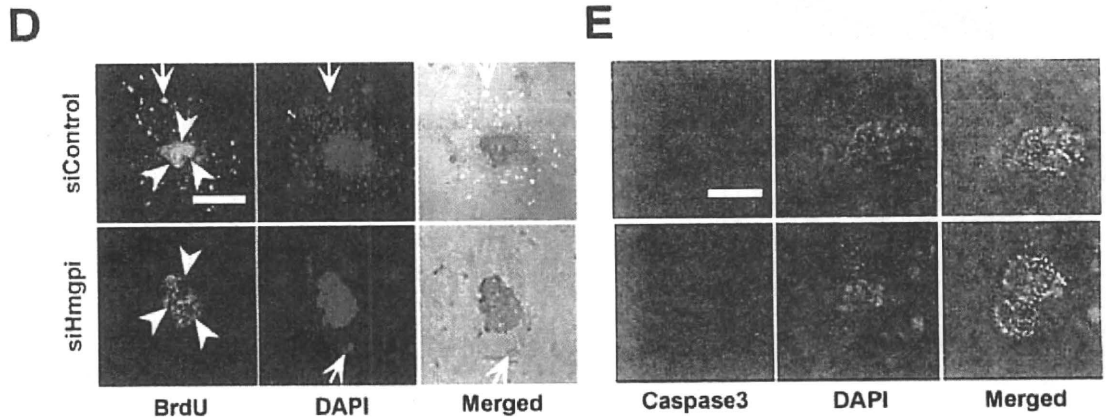


	Tested blastocysts	Attachment (%)	Outgrowth (%)
siControl	51	34 (66.6)	30 (88.2)
siHmgpi	20	15 (75.0)	2 (13.3)
siControl	117	92 (78.6)	80 (86.9)
siHmgpi	13	10 (76.9)	2 (20.0)
siControl	55	49 (89.0)	48 (97.9)
siHmgpi	8	7 (87.5)	1 (14.2)
siControl	41	33 (80.4)	33 (100.0)
siHmgpi	13	10 (76.9)	3 (30.0)



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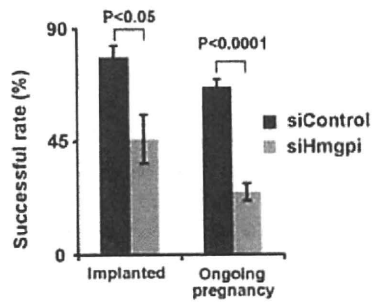

	Transferred embryos	Implanted (%)	Ongoing pregnancy (%)
siControl	30	23 (76.7)	20 (66.7)
siHmgpi	18	6 (33.3)	3 (16.7)
siControl	30	25 (83.3)	18 (60.0)
siHmgpi	32	20 (62.5)	10 (31.2)
siControl	16	13 (81.2)	12 (75.0)
siHmgpi	24	15 (62.5)	7 (29.1)
siControl	20	13 (65.0)	13 (65.0)
siHmgpi	32	8 (25.0)	7 (21.8)

were likewise downregulated in ICM cells of the siHmgpi-injected embryos (Fig. 5B and Supplementary Material, Table S2). Thus, *Hmgpi* is essential for the earliest embryonic development; both ICM and trophectodermal development.

Effect of *Hmgpi* siRNA on *in vivo* and *in vitro* peri-implantation development

To investigate the role of *Hmgpi* in proliferation of the ICM and trophectodermal cells, siHmgpi-injected and siControl-injected embryos were further cultured *in vitro* from the blastocyst stage, and attachment and outgrowth of each embryo on gelatin-coated culture plates was examined. HMGPI expression in siHmgpi-injected embryos was significantly reduced, and immunostaining showed that many colonies of ICM cells in the embryos collapsed during outgrowth culture (Fig. 6A and B). Although the vast majority of ICMs from siControl-injected embryos showed successful attachment (80.3 ± 4.9%) and vigorous outgrowth (96.2 ± 2.7%), those from siHmgpi-injected embryos failed to proliferate or produced only a residual mass (19.3 ± 3.8%) despite successfully attaching (79.0 ± 2.8%) (Fig. 6C; attachment ns; outgrowth, $P < 0.001$). These results implied that *Hmgpi* is essential for proliferation of ICM and trophectodermal cells in peri-implantation development, and for derivation of ES cells.

We then investigated cell proliferation and apoptosis during blastocyst outgrowth. Comparable incorporation of BrdU in blastocyst outgrowths of siHmgpi-injected embryos was less than that of siControl-injected embryos. Proliferation was significantly reduced in ICM-derived cells and dramatically suppressed in trophoblast cells (Fig. 6D). Embryonic fibroblasts were used as a feeder layer in this study and could support ICM cells, thus proliferation should have proceeded regardless of trophectodermal cell support. Therefore, the collapsed ICM-derived colonies in the current experiment were not a secondary effect of reduced proliferation in trophoblast cells, but a direct effect of the siHmgpi-induced decrease in ICM proliferation. Apoptosis was not detected in any cells during blastocyst outgrowth of siHmgpi-injected embryos, based on the absence of active caspase3 (Fig. 6E). Taken together, these findings show that *Hmgpi* is indispensable for proliferation of the ICM and trophectodermal cells in peri-implantation development and for the generation of ES cells.

Finally, we tested whether the experimental blastocysts could develop *in vivo* by transferring siHmgpi-injected and siControl-injected blastocysts into the uterus of pseudopregnant mice. Only 45.8 ± 9.7 and 24.7 ± 3.3% of blastocysts injected with siHmgpi implanted and developed, respectively, whereas most of the siControl-injected embryos showed successful implantation and ongoing development (76.5 ± 4.0 and 66.6 ± 3.3%, respectively) (Fig. 6F; implanted, $P < 0.05$; ongoing pregnancy, $P < 0.0001$). These results confirmed a role for *Hmgpi* in peri-implantation embryonic development.

DISCUSSION

We previously analyzed the dynamics of global gene expression changes during mouse preimplantation development (3). Understanding these preimplantation stages is important for both reproductive and stem cell biology. Many genes showing wave-like activation patterns (e.g. ZGA and MGA) during preimplantation were identified, and any or all of these may contribute to the complex gene regulatory networks. *Hmgpi*, one of the few novel preimplantation-specific genes, is involved in early development, implantation and ES cell derivation.

Structure-based prediction of *Hmgpi* function

Structural information about a protein sometimes hints at functional mechanisms, which remain unknown for *Hmgpi*'s clear role in early embryonic development. The HMG family proteins are abundant nuclear proteins that bind to DNA in a non-sequence-specific manner, influence chromatin structure and enhance the accessibility of binding sites to regulatory factors (17). Based on the number and the type of HMG domains, *Hmgpi* is relevant to the HMGB subfamily, characterized by containing two HMG-box domains ('HMG-box' or 'HMG-UBF_HMG-box'), rather than either the HMGA or HMGN subgroups. *Hmgpi* is also known as *Ubtfl1* in the NCBI gene database, based on sequence similarity to *Ubtf*, a well-known ZGA gene (3,22). *Ubtf*, encoding a SANT domain and six HMG-box domains, functions exclusively in RNA polymerase I (Pol I) transcription (23) and acts through its multiple HMG boxes to induce looping of DNA, which creates a nucleosome-like structure to modulate tran-

Figure 6. Function of *Hmgpi* in peri-implantation development. (A) Blastocyst outgrowth and alkaline phosphatase (AP) activity in the siHmgpi-injected and siControl-injected embryos, carried out according to a standard procedure (42). Representative images of phase-contrast microscopy for blastocyst outgrowth and fluorescent immunocytochemistry for AP are shown. Scale bar = 100 μM. (B) Confocal microscopy images of blastocyst outgrowth for the siHmgpi-injected and siControl-injected embryos, stained with antibodies to Hmgpi and Oct4. Nuclei are shown by DAPI staining. Scale bar = 100 μM. (C) Successful rate of blastocyst outgrowth for siHmgpi-injected and siControl-injected embryos. Successful outgrowth in this assay was indicated by the presence of proliferating cells after 6 days in culture. The experiment was repeated four times. (D) BrdU incorporation assay for blastocyst outgrowth of the siHmgpi-injected and siControl-injected embryos. Cell proliferation was determined by BrdU incorporation (ICM: arrowhead, trophectodermal cells: arrow). The trophectodermal component contained few cells and BrdU incorporation was confined to the ICM core; however, cell proliferation was reduced in the blastocyst outgrowth of siHmgpi-injected embryos compared with that of the siControl-injected embryos. Nuclei are shown by DAPI staining. Scale bar = 100 μM. (E) Immunocytochemistry with an anti-caspase3 antibody in blastocyst outgrowth of the siHmgpi-injected and siControl-injected embryos. Apoptotic cells were not apparent in the blastocyst outgrowth of either injected embryo. Nuclei are shown by DAPI staining. Scale bar = 100 μM. (F) Successful rate of siHmgpi-injected and siControl-injected embryo transfer. We transferred 3.5 dpc blastocysts into the uteri of 2.5 dpc pseudopregnant ICR female mice. The pregnant ICR mice were sacrificed on day 12.5 of gestation and the total numbers of implantation sites and of live and dead embryos/fetuses were counted. The experiment was repeated four times.

scription of the 45S precursor of ribosomal RNA (rRNA) by Pol I (24,25). Because the association of UBTF with rRNA genes *in vivo* is not restricted to the promoter and extends across the entire transcribed portion, UBTF promotes the formation of nucleolar organizer regions, indicative of 'open' chromatin (26). Based on the sequence similarity between UBTF and HMGPI, HMGPI might also bind to DNA in a non-specific manner, and modulate chromatin during peri-implantation when dynamic chromatin change is essential.

Alternatively, HMGPI may act as a cytokine during preimplantation development in a similar manner to HMGB1. HMGB proteins are found primarily in the cell nucleus, but also to varying extents in the cytosol (27,28), and have been suggested to shuttle between compartments (17). HMGB1 is indeed passively released from nuclei upon cell death and actively secreted as a cytokine (29), and the addition of recombinant HMGB1 into culture medium enhances *in vitro* development of mouse zygotes to the blastocyst stage in the absence of BSA supplementation (30). Although HMGPI failed to be detected in culture media after *in vitro* culture of preimplantation embryos or ES cells in this study (data not shown), two different modes of Hmgpi action, chromatin modulator and secreted mediator, should be taken into consideration as discussed later.

Role of *Hmgpi* during peri-implantation

The HMGPI protein was first detected in 4-cell embryos and then abundantly expressed in 8-cell embryos, morulae, ICM, trophectoderm and ES cells. Although *Hmgpi* transcription peaked at the 4-cell stage, the most dramatic siRNA effect appeared at the blastocyst and subsequent stages. This discrepancy between temporal expression and phenotype is attributed to three possible mechanisms. First, protein expression is generally delayed from transcription; indicated here by the *Hmgpi* transcripts and HMGPI protein expression peaking at the 4-cell stage and blastocyst stage, respectively. Similarly, *Stella* (31) and *Pms2* (32) are maternal-effect genes, but do not cause developmental loss until later preimplantation stages. A second possibility is the incompleteness of siRNA knockdown. One limitation of such knockdown experiments is the potential variability in levels of silencing of a target gene, which could in turn underlie the observed phenotypic variability in the present study. Embryos with complete suppression of Hmgpi may exhibit developmental arrest at earlier stages (e.g. at the morula stage), while those with less suppression may not display a phenotype until the later stages (e.g. at the implantation stage). Ideally, the suppression level of each embryo could be experimentally analyzed to correlate with the phenotype. The third possibility is spatial translocation of HMGPI protein in the blastocyst cells. The HMGPI expression pattern indicated differential spatial requirements during early embryogenesis, supported by the apparent ability of HMGPI to shuttle between the nucleus and the cytoplasm; the cytoplasmic HMGPI observed from the 4-cell to morula stages and the nuclear HMGPI in blastocysts and ES cells could have different functions. A bipartite nuclear localization signal (NLS) peptide (FKKEKEDFQKKMRQFKK) similar to NLS of HMGN2/HMG-17 (33) is also present in the HMGPI sequence. Thus, the nuclear HMGPI in blastocysts and ES cells might exert a critical transcriptional role to regulate gene expression essential for peri-implantation development. Indeed, the siHmgpi-induced knockdown of *Hmgpi* expression downregulated *Cdx2* in trophectodermal cells and *Oct4* and *Nanog* in ICM cells, with subsequently reduced proliferation of trophectodermal cells and ICM-derived cells during blastocyst outgrowth.

Genes indispensable for derivation of ES cells

Like *Hmgpi*, *Zscan4* is another exclusively zygotic gene not expressed at any other developmental stage (13). *Zscan4* is a putative transcription factor harboring a SCAN domain and zinc finger domains, and transcribed not only in preimplantation embryos but also in ES cells (13). Reduction of *Zscan4* by RNA interference showed a phenotype similar to that induced by *Hmgpi* knockdown: developmental deterioration at the preimplantation stages, especially cleavage pause at 2-cell stage, and failure in blastocyst outgrowth, ES-cell derivation and implantation. Thus, a preimplantation-specific gene expression pattern could indicate a function in ES-cell derivation and/or maintenance. Indeed, *Hmgpi* was also expressed in entire ES colonies, whereas *Zscan4* shows a peculiar mosaic expression pattern in undifferentiated ES cell colonies. Furthermore, the *Hmgpi* gene is highly expressed in ES cells, but not in EC cells; *Hmgpi* is thus eligible as a putative ECAT (ES cell-associated transcript), whose ESTs are overrepresented in cDNA libraries from ES cells compared with those from somatic tissues and other cell lines including EC cells (34). It is also likely that *Hmgpi* is expressed in iPS cells, based on *in silico* analyses of expression profiles [NCBI GEO database, e.g. GSE10806 (35)]. Thus, *Hmgpi* is likely to have a role in maintaining pluripotent cells, since the ECATs such as *Nanog*, *Eras* and *Gdf3* are required for pluripotency and proliferation of ES cells (34,36,37). In the current study, *Hmgpi* was indeed involved in blastocyst outgrowth of ICM cells. On the other hand, several genes including ECAT members have been implicated in trophectodermal development as well as in early embryonic development. Like *Hmgpi* that was expressed in both ICM cells and trophectodermal cells, *Dnmt3l/Ecat7* has a role in embryonic and extra-embryonic tissues in early developmental stages. *DNMT3L* is recruited by *DNMT3A2* to chromatin (38) to function in DNA methylation in ES cells, and defects in maternal *DNMT3L* induce a differentiation defect in the extra-embryonic tissue (39). The reduced CDX2 expression in blastocysts and poor BrdU incorporation during blastocyst outgrowth following siHmgpi knockdown suggested the potential involvement of *Hmgpi* in trophectodermal development.

In summary, *Hmgpi* is required early on in mammalian development to generate healthy blastocysts that implant successfully and produce ES cells. HMGPI translocates into the nucleus from cytoplasm at the blastocyst stage, which is importantly a turning point of early embryonic development when DNA-methylation levels are at their lowest and implantation takes place. The nuclear HMGPI in blastocysts and ES cells is expected to act as a transcription factor to regulate gene expression networks underlying the generation, self-renewal and maintenance of pluripotent cells. Because E7 embryos have already stopped expressing *Hmgpi*, it is likely