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

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Mining gene-expression-profiling data identified a novel gene that is specifically expressed in preimplantation embryos. \textit{Hmgpi}, a putative chromosomal protein with two high-mobility-group boxes, is zygotically transcribed during zygotic genome activation, but is not transcribed postimplantation. The \textit{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 \textit{Hmgpi} transcript levels caused developmental loss of preimplantation embryos and implantation failures. Furthermore, reduction of \textit{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 \textit{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 \textit{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 \textit{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

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and Myc (4). These iPSC 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 iPSCs. 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 iPSC 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 iPSCs 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 Ubtfl-like 1 (Ubtfl1) based on aa sequence similarity (36% identity and 58% positives by BLAST) to Ubtfl-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 preimplantation-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...
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 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).
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.
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.
nuclear and cytoplasmic fractions of ES cells (Fig. 3C).

and immunoblotting confirmed HMGPI expression in both differentiated ES cells in a colony also expressed HMGPI (Fig. 3B), ES cells, we found that almost all the Oct4-positive undifferentiated cells, with scant amounts detected in the cytoplasm HMGPI was expressed in the nuclear region of most outgrowths (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).

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).

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 Ubf1, Hmgb1, Hmgb2 and Hmgb3. Although Ubf1, 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 ± 1.3% of siHmgpi-injected embryos became blastocysts, while 94.1 ± 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 blastocoeals, suggesting impairment of trophoectodermal 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 extra-embryonic development, was particularly and markedly down-regulated in trophoectodermal cells, while Nanog and Oct4...
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 trophectodermal 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 trophectodermal 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 HMBG 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 UbtfH1 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. 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 RNA 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, trophoderm 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 (FKKEKEDFQKMRQFKK) 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 trophodermal cells and Oct4 and Nanog in ICM cells, with subsequently reduced proliferation of trophodermal 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 pre-implantation 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 iP 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 trophodermal development as well as in early embryonic development. Like Hmgpi that was expressed in both ICM cells and trophodermal cells, Dmnt3l/Ecat7 has a role in embryonic and extraembryonic 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 extraembryonic 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 trophodermal 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...
that Hmgpi stage-specifically regulates a set of genes that drive peri-implantation development. It will be valuable to identify both cofactors that bind HMGPI and recognize specific DNA sequences, as well as genes that are regulated by Hmgpi using ES cells. A better understanding of the Hmgpi transcriptional network will also improve culture methods for healthy blastocysts and for generating, maintaining and differentiating ES cells.

MATERIALS AND METHODS

Identification of the mouse Hmgpi gene by in silico analysis

Preimplantation-specific genes were identified based on global gene expression profiling of oocytes and preimplantation embryos (3,40) and expressed sequence tag (EST) frequencies in the Unigene database. SMART (19) was used for domain prediction analysis. Orthologous relationships between HMG family genes were identified from phylogenetic-tree amino acid sequences determined by a sequence distance method and the Neighbor Joining (NJ) algorithm (41) using Vector NTI software (Invitrogen, Carlsbad, CA, USA).

Collection and manipulation of embryos

Six- to 8-week-old B6D2F1 mice were superovulated by injecting 5 IU of pregnant-mare serum gonadotropin (PMS; Calbiochem, La Jolla, CA, USA) followed by 5 IU of human chorionic gonadotropin (HCG; Calbiochem) 48 h later. The Institutional Review Board of the National Research Institute for Child Health and Development, Japan granted ethics approval for embryo collection from the mice. Unfertilized eggs were harvested 18 h after the HCG injection by a standard published method (42), and the cumulus cells were removed by incubation in M2 medium (EmbryoMax M-2 Powdered Mouse Embryo Culture Medium; Millipore, Billerica, MA, USA) supplemented with 300 μg/ml hyaluronidase (Sigma-Aldrich, St Louis, MO, USA). The eggs were then thoroughly washed, selected for good morphology and collected. Fertilized eggs were also harvested from mated superovulated mice in the same way as unfertilized eggs and embryos with two pronuclei (PN) were collected to synchronize in vitro embryo development. Fertilized eggs were cultured in synthetic oviductal medium enriched with potassium (EmbryoMax KSOM Powdered Mouse Embryo Culture Medium; Millipore) at 37°C in an atmosphere of 95% air/5% CO2. Cultured blastocysts were transferred into pseudo-pregnant recipients as described previously (42). We transferred 3.5 dpc blastocysts into the uteri of 2.5 dpc pseudopregnant ICR female mice. RNA interference experiments were carried out by microinjecting <10 pl (25 ng/μl) of oligonucleotides (siHmgpi and siControl) into the cytoplasm of zygotes. The optimal siRNAs were determined by testing different concentrations (5, 10, 25 and 50 ng/μl) of three siRNAs (PE Applied Biosystems, Foster City, CA, USA), resuspended and diluted with the microinjection buffer (Millipore). Their target sequences are listed in Supplementary Material, Table S3. More than 10 independent experiments were performed to study the effect of Hmgpi knockdown on preimplantation development and implantation.

Culture of ES cells and blastocyst outgrowth

A mouse ES cell line (B6/129ter/sv line) was first cultured for two passages on gelatin-coated culture dishes in the presence of leukemia inhibitory factor (LIF) to remove contaminating feeder cells. Cells were then seeded on gelatin-coated 6-well plates at a density of 1–2 x 10^5/well (1–2 x 10^3/cm²) and cultured for 3 days in complete ES medium: KnockOut DMEM (Invitrogen) containing 15% KnockOut Serum Replacement (KSR; Invitrogen), 2000 U/ml ESGRO (mLIF; Chemicon, Temecula, CA, USA), 0.1 mM non-essential amino acids, 2 mM GlutaMax (Invitrogen), 0.1 mM beta-mercaptoethanol (2-ME; Invitrogen) and penicillin/streptomycin (50 U/50 μg/ml; Invitrogen). Blastocyst outgrowth experiments were carried out according to a standard procedure (42). In brief, zona pellucidae of blastocysts at 3.5 dpc were removed using acidic Tyrode’s solution (Sigma). The blastocysts were cultured individually in the ES medium on gelatinized chamber slides at 37°C in an atmosphere of 5% CO2. The cultured cells were examined and photographed daily. Alkaline phosphatase activity was measured using a specific detection kit (Vector Laboratories, CA, USA) after 6 days in culture. Four independent experiments were performed.

Immunostaining of oocytes and preimplantation embryos

Samples were fixed in 4% paraformaldehyde (Wako Pure Chemical, Osaka, Japan) with 0.1% glutaraldehyde (Wako) in phosphate-buffered saline (PBS) for 10 min at room temperature (RT), and then permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 30 min. Immunocytochemical staining was performed by incubating the fixed samples with primary antibodies for 60 min, followed by secondary antibodies for 60 min. A polyclonal antibody to mouse HMPGI was raised in rabbits against three synthesized peptides designed according to sequence specificity, homology between mouse and human HMGPI, antigenicity, hydrophilicity and synthetic suitability (i) CIQGHHDGAQSSRQDFTD, (ii) CMSMSGG, (iii) ESPRTVSSDMKFQGC (Medical & Biological Laboratories Co, Nagoya, Japan). The anti-HMPGI was used at 1:300 dilution, followed by Alexa Fluor 464 goat anti-rabbit IgG (Molecular Probes, Invitrogen) as the secondary antibody. The anti-Histone H2B antibody (Medical & Biological Laboratories Co, Nagoya, Japan) was used at 1:300 dilution as positive control of nuclear staining, followed by Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Invitrogen) as the secondary antibody. Blastocysts were immunostained using a monoclonal anti-Oct4 antibody (mouse IgG2b isotype, 200 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-Nanog antibody (ReproCELL, Tokyo, Japan), mouse monoclonal anti-Cdx2 antibody (CELL MARQUE, Rocklin, CA, USA), mouse monoclonal anti-BrdU antibody (Santa Cruz) and rabbit monoclonal anti-active caspase 3 (Abcam) antibody, all diluted at 1:50–300. The appropriate secondary antibodies (IgG) were diluted at 1:300 and supplied by Molecular Probes/Invitrogen: goat anti-rabbit IgG conjugated with Alexa Fluor 546 and goat anti-mouse IgG(H + L) conjugated with Alexa Fluor 488. The cellular DNA (nuclei) was stained with 4’,6-diamidino-2-phenylindole (DAPI; Wako; diluted...
The cells were then washed with PBS and viewed by laser confocal microscopy (LSM510, Zeiss). For HMGPI immunostaining, all samples 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 HMGPI expression at different developmental stages. The other molecules in blastocysts and outgrowth were viewed and imaged as for the HMGPI expression.

Immunocytchemistry of blastocyst outgrowths and ES cells
Cultured ES cells and blastocyst outgrowths were fixed with 4% paraformaldehyde for 10 min at 4°C, treated with 0.1% Triton X-100 (Sigma) in PBS for 15 min at RT, and then incubated for 30 min at RT in protein-blocking solution consisting of PBS supplemented with 5% normal goat serum (Dako, Glostrup, Denmark). The samples were then incubated overnight with the primary antibodies to OCT4, HMGPI, BrdU or active caspase 3 in PBS at 4°C. The cells were then extensively washed in PBS and incubated at RT with Alexa Fluor 488 goat anti-mouse IgG1 (anti-OCT4 and anti-BrdU antibodies, diluted 1:300; Molecular Probes) or Alexa Fluor 546 goat anti-rabbit IgG(H+L) (anti-HMGPI and anti-caspase 3 antibodies, diluted 1:300), and nuclei were counterstained with DAPI for 30 min. To prevent fading, cells were then mounted in Dako fluorescent mounting medium (Dako).

Incorporation of bromodeoxyuridine (BrdU)
E3.5 blastocysts and blastocyst outgrowths were cultured for 16 h in KSOM and ES medium, respectively, supplemented with 10 μM BrdU (Sigma). Samples were then fixed in 4% paraformaldehyde for 20 min, washed in PBS and then treated with 0.5 M HCl for 30 min.

RNA extraction and real-time quantitative reverse transcriptase (qRT–PCR)
Embryos for qRT–PCR analysis were collected at 18 h post-hCG and cultured as described above. They were harvested at 0.5, 1.25, 1.75, 2.25, 2.75 and 3.75 dpc to obtain fertilized eggs 2-cell, 4-cell, 8-cell, morula and blastocyst embryos, respectively. Three subsets of 10 and 50 synchronized and intact embryos were transferred in PBS supplemented with 3 mg/ml polyvinylpyrrolidone (PVP) and stored in liquid nitrogen. Total RNA from 10 and 50 embryos was extracted using the PicoPure RNA Isolation Kit (Arcturus, La Jolla, CA, USA). The reverse transcription reaction, primed with polyA primer, was performed using Superscript III reverse transcriptase (Invitrogen) following the manufacturer’s instructions. Total RNA isolated was reverse transcribed in a 20 μl volume. The resulting cDNA was quantified by qRT–PCR analysis using the SYBR Green Realtime PCR Master Mix (Tóyobo, Osaka, Japan) and ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) as described previously (43). An amount of cDNA equivalent to 1/2 an embryo was used for each real-time PCR reaction with a minimum of three replicates, with no-RT and no-template controls for each gene. Data were normalized against H2afz by the ΔΔCt method (44). PCR primers for the genes of Hmgpi, H2afz and Gapdh were listed in Supplementary Material, Table S4. Calculations were automatically performed by ABI software (Applied BioSystems). For alpha-amanitin studies, fertilized eggs were first harvested at 18 h post-hCG, instead of eggs already advanced to the two-pronucleus stage. After 3 h of incubation, eggs that carried both male and female pronuclei were selected at 21 h post-hCG and randomly assigned to two experimental groups: with and without addition of alpha-amanitin to the culture medium. The eggs were further cultured in KSOM at 37°C in an atmosphere of 5% CO2 until the specified time point (32, 43 and 54 h post-hCG). Embryos used for alpha-amanitin studies and RNA interference experiments were subjected to qRT–PCR as described for the normal preimplantation embryos.

Immunoblot analysis
Protein samples from embryos were solubilized in Sample Buffer Solution without 2-ME (Nacalai Tesque, Kyoto, Japan), resolved by NuPAGE Novex on Tris-acetate mini gels (Invitrogen), and transferred to Immobilon-P transfer membrane (Millipore). The membrane was soaked in protein blocking solution (Blocking One solution, Nacalai) for 30 min at RT before an overnight incubation at 4°C with primary antibody, also diluted in blocking solution. The membrane was then washed three times with TBST (Tris-buffered saline with 0.1% Tween-20), incubated with a horseradish peroxidase-conjugated secondary antibody (0.04 μg/ml) directed against the primary antibody for 60 min, and washed three times with TBST. The signal was detected by enhanced chemiluminescence (SuperSignal West Dura Extended Duration Substrate, Thermoscientific, Rockford, IL, USA) following the manufacturer’s recommendations. The intensity of the band was quantified using NIH Image J software. Briefly, the signal was outlined and the mean intensity and background fluorescence were measured. The specific signal was calculated by dividing the band intensities for HMGPI by those for actin.

Statistical analysis
Differences between groups were evaluated statistically using Student’s t-test or ANOVA, with P-values < 0.05 considered significant.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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