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—Original Article—

Involvement of histone H2B monoubiquitination in the regulation of mouse preimplantation development

Masatoshi OOGA[1)], Masataka G. SUZUKI[1)] and Fugaku AOKI[1)]

[1)]*Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Chiba 277-8562, Japan*

Abstract. Histone H2B monoubiquitination (H2Bub1) plays an important role in developmental regulation in various vertebrate species. However, the role of H2Bub1 in mammalian preimplantation development remains unclear. In the present study, we examined the role of H2Bub1 in the regulation of mouse preimplantation development. Based on immunocytochemical analysis using an anti-H2Bub1 antibody, no H2Bub1 signal was detected in the metaphase chromosomes of unfertilized oocytes or the pronuclei of early 1-cell stage embryos, but a weak signal was observed in late 1-cell stage embryos. The signal increased after cleavage into the 2-cell stage, and thereafter a strong signal was observed until the blastocyst stage. To assess the significance of H2Bub1 in the regulation of preimplantation development, RNF20 (an H2B-specific ubiquitin E3 ligase) was knocked down using small interfering RNA (siRNAs). In embryos treated with siRNA, the levels of *Rnf20* mRNA and H2Bub1 decreased at the 4-cell and morula stages. Although these embryos developed normally until the morula stage, only one-third developed into the blastocyst stage. These results suggested that H2Bub1 is involved in the regulation of preimplantation development.

Key words: H2B monoubiquitination, Mouse embryo, Preimplantation development

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Monoubiquitination of histone H2B at the 120th lysine residue (H2Bub1) in mammals alters the chromatin structure and plays a role in developmental regulation in various multicellular organisms. H2Bub1 is catalyzed by Rad6p and Bre1p, a ubiquitin-conjugating E2 enzyme and ubiquitin E3 ligase, respectively, in yeast [1–5]. Rad6p is involved in the ubiquitination of various proteins [6–9]. The substrate specificity of Rad6p towards histone H2B is determined by Bre1p, which recruits Rad6p to the target chromatin [6, 10–12]. In *Drosophila*, H2B is monoubiquitinated by dBre1, the ortholog of yeast Bre1p. The decrease in H2Bub1 levels caused by a mutation in *dBre1* results in the typical phenotype of Notch signaling disruption [13, 14]. In addition, an increase in H2Bub1 levels caused by a mutation in the *Drosophila* H2B ubiquitin protease *scrawny* increased the expression of Notch target genes, resulting in premature differentiation and a reduction in the number of stem cells in the germ line, follicles and intestine [13, 15]. In *Arabidopsis*, a deficiency in H2Bub1 caused by a mutation in *Hub1* and *Hub2* (plant orthologs of yeast *Bre1*) decreased seed dormancy and plant size and caused precocious flowering [13, 16, 17]. The increase in H2Bub1 levels caused by a mutation in ubiquitin-specific protease 26 also resulted in the early-flowering phenotype and smaller leaves [18]. *RNF20* and *RNF40* are human orthologs of yeast *Bre1*. Reduction of H2Bub1 using small interfering RNA (siRNAs) targeting these two genes resulted in decreased expression of *HOX* genes [19]. On the other hand, increase of H2Bub1 by overexpression of *RNF20* caused abnormally stimulated expression of *HOX* genes [19]. Knockdown of *Rnf20* led to insufficient upregulation of differentiation-related genes during mouse ES cell differentiation, resulting in deficient neural differentiation [20]. Numerous reports have shown that H2Bub1, which is mediated by Bre1p and its orthologs, plays pivotal roles in developmental regulation. However, to the best of our knowledge, little is known about the role of H2Bub1 in the regulation of preimplantation development.

Preimplantation development is driven by dynamically altered gene expression. In mice, transcription from the zygotic genome is initiated at the mid-1-cell stage after fertilization [21]. Although the transcriptional activity is very low during this stage, it increases gradually until the early 2-cell stage, after which a burst of transcription occurs at the mid to late 2-cell stage [21, 22]. Subsequently, active transcription occurs constantly until the blastocyst stage. The gene expression profile is also significantly altered as preimplantation development progresses [23–25]. Since H2Bub1 plays an important role in the regulation of active transcription in various types of cells and tissues [11, 20, 26–28], we hypothesized that H2Bub1 is involved in the regulation of preimplantation development through the control of gene expression.

In this report, we investigated the roles and dynamics of H2Bub1 in the regulatory mechanisms of preimplantation development. Our results showed that H2Bub1 was absent in unfertilized oocytes and early 1-cell stage embryos but present at low levels in late 1-cell stage embryos. It was detected at high levels during subsequent preimplantation development. Knockdown of *Rnf20* using siRNAs decreased the rate of development to the blastocyst stage. In addition, the number of cells in embryos that could develop to the

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Correspondence: F Aoki (e-mail: aokif@k.u-tokyo.ac.jp)

blastocyst stage decreased significantly. These results suggested that H2Bub1 is involved in the regulatory mechanisms of preimplantation development.

Materials and Methods

Collection and culture of oocytes and embryos

To collect unfertilized oocytes, cumulus-oocyte complexes (COCs) were obtained from 3-week-old BDF1 (B6D2F1) mice that had been injected with equine chorionic gonadotropin (ASKA Pharmaceutical, Tokyo) followed 48 h later by 5 IU human chorionic gonadotropin (ASKA Pharmaceutical). The COCs were transferred into human tubal fluid (HTF) medium [29] supplemented with 10 mg/ml BSA. Hyaluronidase was added to HTF medium at a final concentration of 300 μg/ml (Sigma-Aldrich, St. Louis, MO, USA) to remove cumulus cells. After being washed, the denuded oocytes were used for reverse transcription polymerase chain reaction (RT-PCR) or immunocytochemistry.

For *in vitro* fertilization, the COCs were transferred into HTF medium and inseminated with spermatozoa that had been collected from the cauda epididymis of mature male ICR mice (SLC, Shizuoka, Japan) and incubated in HTF medium supplemented with 1% BSA at 38 C for 2 h. Six hours after insemination, the fertilized oocytes were washed and then cultured in potassium simplex optimized medium (KSOM [30]) containing 3 mg/ml BSA.

All of the procedures using animals were reviewed and approved by the University of Tokyo Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Immunocytochemistry

Oocytes and embryos were fixed with 3.7% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min at room temperature. After being washed with PBS containing 1% BSA (1% BSA/PBS), the cells were permeabilized with 0.2% Triton X-100 in PBS for 30 min and then washed with PBS containing 0.05% Tween 20. The cells were incubated with 4 N HCl containing 0.1% Triton X-100 for 10 min at room temperature and then transferred into 0.1 M Tris-HCl (pH 8.5) containing 0.02% Triton X-100. After a 30-min incubation, the cells were washed in 1% BSA/PBS and then incubated with an antibody against H2Bub1 (Medimabs, Mont-Royal, QC, Canada; #MM-0029-p; 1:200 dilution with 1% BSA/PBS) overnight at 4 C. After washing with 1% BSA/PBS, cells were incubated with a fluorescent-labeled secondary antibody, namely, Alexa Fluor 488 conjugated anti-mouse IgG (Life Technologies, Carlsbad, CA, USA, #A11001; 1:100 dilution). Cells were mounted on a glass slide in Vectashield antibleaching solution (Vector Laboratories, Burlingame, CA, USA) containing 3 μg/ml 4',6-diamino-2-phenylindole (DAPI; Dojindo, Kumamoto). The fluorescence signals were detected using an LSM 5 Exciter confocal microscope system (Carl Zeiss, Oberkochen, Germany).

Semi-quantitative RT-PCR

Total RNA was extracted from 12 oocytes and embryos using ISOGEN (Nippon Gene, Tokyo, Japan) and subjected to reverse transcription using an Oligo dT primer and PrimeScript RT-PCR kit according to the manufacturer's instructions (Takara, Ohtsu, Japan). As an external control, 50 pg rabbit α-globin mRNA purchased from Invitrogen was added prior to total RNA isolation. The obtained cDNA was used for PCR with Ex Taq DNA polymerase (Takara). The primers used for PCR were as follows: *Rnf20*, 5′-tgcagatgacctcaaagcac-3′ (forward) and 5′-ttcatcacacttgggcacat-3′ (reverse), and rabbit α-globin, 5′-gtgggacaggagcttgaaat-3′ (forward) and 5′-gcagccacggtggcgagtat-3′ (reverse). PCR was performed by denaturation at 95 C for 2 min followed by 37 (*Rnf20*) or 24 cycles (rabbit α-globin) of 95 C for 20 sec (*Rnf20*) or 30 sec (rabbit α-globin) (denaturation), 60 C for 20 sec (annealing) and 72 C for 30 sec (elongation). To confirm knockdown of *Rnf20,* the primer pair 5'-gagtttgagcagacccttgc-3' (forward) and 5'-gcagacgtgtcttgttcagg-3' (reverse) or 5'- gcatcacac-catgtctcagg-3' (forward) and 5'-cacccgctctaggacttcag-3' (reverse) was used. These primers were designed to amplify the region near the target sites of siRNAs against *Rnf20*. The PCR conditions for these primer pairs were as follows: 95 C for 2 min, followed by 32 cycles of 95 C for 30 sec (denaturation), 63 C for 30 sec (annealing) and 72 C for 30 sec (elongation). To analyze the effects of *Rnf20* knockdown on the expression of genes encoding histones, RNA was extracted from 25 embryos at the morula stage. The isolated RNA was treated with DNase I, after which the cDNA was reverse transcribed using random six-mer primers. After reverse transcription, cDNA was subjected to PCR using Ex Taq. The primers for PCR were as follows: *hist1h2ab*, 5'-ctaaggccaagacccgctc-3' (forward) and 5'-tcgccagattacttccccttg-3' (reverse); *h2afy*, 5'- gctagcgaagaagcgag-gat-3' (forward) and 5'-ccccttttcttgcctcctgtc-3' (reverse); *hist1h2bb*, 5'-tcgtgaacgacatcttcgag-3' (forward) and 5'-ccctacgagctcacttggag-3' (reverse); *hist1h2bg*, 5'- gcttgtttctaccatgcccg-3' (forward) and 5'-atggtcgagcgcttgttgta-3' (reverse); and *hist2h2bb*, 5'-atcacttcccggga-gatcca-3' (forward), 5'-agccttttgggtaaagccga-3' (reverse). PCR was performed by denaturation at 95 C for 2 min followed by 32 (*h2afy*), 35 (hist1h2bb, hist1h2bg and hist2h2bb) or 38 cycles (*hist1h2ab*) of 95 C for 30 sec (denaturation), 60 C (*hist1h2ab, hist1h2bb, hist1h2bg* and *hist2h2bb*) or 63 C (*h2afy*) for 30 sec (annealing) and 72 C for 30 sec (elongation). PCR products were separated on 2% agarose gels and stained with ethidium bromide. The gel image was obtained using a DT-20MP UV illuminator (ATTO, Tokyo).

Microinjection of siRNAs

Stealth RNAi^{TM} small interfering RNAs (siRNAs) against *Rnf20* (siRnf20 #1: 5'-cagucacaguucucuguccuguaua, and siRnf20 #2: 5'-caggaguucuguaaguugcagggua-3') or GFP (siGFP: 5'-ccacuac-cugagcacccaguccgcc-3') were purchased from Life Technologies. Microinjection was performed on an inverted microscope (Eclipse TE300; Nikon, Tokyo, Japan) using a micromanipulator (Narishige) and microinjector (IM300; Narishige). To remove surrounding cumulus cells at 2 h post insemination, the zygotes were treated with hyaluronidase in HTF for 5 min, followed by repeated pipetting using narrow glass capillaries. Approximately 5 pl siRNA (10 μM) was microinjected into the cytoplasm of zygotes in Hepes-buffered KSOM using narrow glass capillaries (Harvard Apparatus; GC100 TF-10).

Immunosurgery of blastocysts

Embryos were collected at the blastocyst stage 96 h after insemination and treated with anti-mouse red blood cells (Rockland, Boyertown,

PA, USA, #110-4139; 1:100) in α-MEM (Life Technologies) for 30 min at 37 C. The cells were washed and incubated in α-MEM for 5 min. To stain DNA in the trophectoderm, the embryos were incubated in α-MEM containing guinea pig complement sera (Sigma-Aldrich, Cat# S-1639; diluted 1:100) and propidium iodide (PI; Sigma-Aldrich, P4864; 1:100) for 10 min, after which the embryos were washed in α-MEM. Embryos were treated with hypotonic solution (0.9% sodium citrate containing 0.3% polyvinylpyrrolidone) and then fixed in a mixture of methanol, acetic acid, and sterilized water (5:1:4). The fixed embryos were adhered to the surface of the glass slide by dripping a solution of methanol and acetic acid (3:1) on them. The glass slides were dried and consecutively soaked in mixtures of methanol and acetic acid (3:1) and methanol, acetic acid and sterilized water (3:3:1) for 30 min and 1 min, respectively. The embryos were enclosed in Vectashield containing 3 μg/ml DAPI with cover slips. Images of the embryos were obtained using an LSM 5 Exciter confocal microscope system, after which the numbers of nuclei stained purple (trophectoderm) and blue (ICM) were counted using an LSM Image Browser (Carl Zeiss).

Results

Alteration of H2Bub1 during preimplantation development

To examine the dynamics of H2Bub1 during preimplantation development, we performed immunocytochemistry using an anti-H2Bub1 antibody. H2Bub1 was not detected in the metaphase chromosomes of MII oocytes or the pronuclei of early 1-cell stage embryos (Fig. 1). However, a weak fluorescent signal of H2Bub1 was observed at the late 1-cell stage, and the signal intensity increased after cleavage into the 2-cell stage. The intense H2Bub1 signal was maintained during subsequent preimplantation development until the blastocyst stage (Fig. 1).

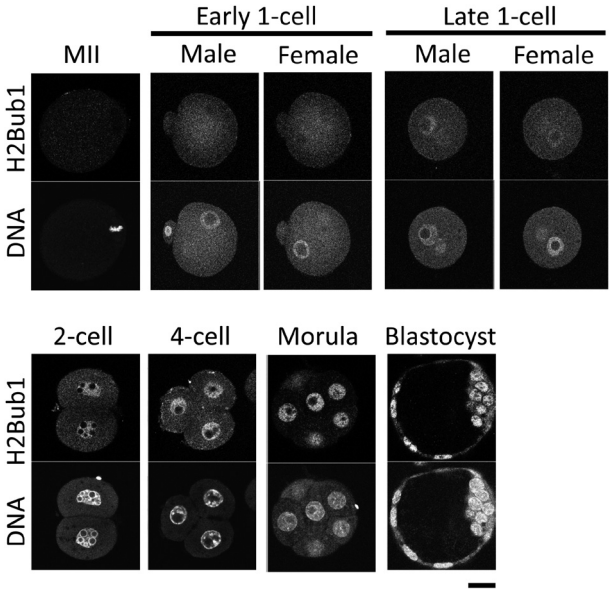

Fig. 1. H2Bub1 in mouse preimplantation embryos. The MII stage oocytes (MII) and preimplantation embryos were immunostained with anti-ubiquitinated H2B (H2Bub1) antibody. Embryos at the early 1-cell, late 1-, 2- and 4-cell, morula and blastocyst stages were collected 6, 12, 28, 45, 70 and 96 h after insemination, respectively. Images of two confocal planes are shown for 1-cell stage embryos to clearly reveal the male and female pronuclei. More than three independent experiments (in which more than 20 oocytes/embryos were observed) were performed for each stage of oocyte and preimplantation embryo. DNA was stained with 4',6-diamino-2-phenylindole (DAPI). Similar results were obtained in each experiment, and representative images are shown. Bar = 20 μm.

H2Bub1 plays a role in preimplantation development

As described above, H2Bub1 appeared after fertilization and was maintained at a high level until the blastocyst stage. Based on this result, we assessed the significance of H2Bub1 for the progression of preimplantation development. To accomplish this, we knocked down *Rnf20*, a histone H2B-specific E3 ubiquitin ligase, and examined whether the decrease in H2Bub1 caused by *Rnf20* knockdown affected preimplantation development. In previous studies, knockdown of *Rnf20* significantly decreased H2Bub1 levels in several different types of cells [20, 31–33].

First, we examined the expression of *Rnf20* in preimplantation embryos. RT-PCR analysis revealed that *Rnf20* mRNA was expressed at a constant level throughout preimplantation development (Fig. 2A). To suppress *Rnf20* expression, siRNAs against *Rnf20* were injected into 1-cell stage embryos. The level of *Rnf20* mRNA was reduced efficiently by the two siRNAs (siRnf20 #1 and #2) at the 4-cell stage, and the level of H2Bub1 was decreased in 4-cell and morula stage embryos (Fig. 2B and C), although the siRNAs could not reduce the H2Bub1 level at the 1- and 2-cell stages (data not shown). These results indicate that *Rnf20* is an essential factor regulating H2Bub1 in preimplantation embryos, as observed in several types of cells. The *Rnf20*-knocked down embryos developed normally until the morula stage. However, only one-third developed to the blastocyst stage, whereas more than 70% of control embryos injected with GFP siRNA developed to the blastocyst stage (Fig. 3). Among the embryos that had developed to the blastocyst stage, the number of cells was significantly smaller in the embryos injected with *Rnf20* siRNAs than in the control embryos ($P < 0.05$ and < 0.01 for siRnf20 #1 and siRnf20 #2, respectively, based on the Student's *t*-test) (Fig. 4A). The decrease in cell number was observed both in the inner cell mass and trophectoderm, which were discriminated based on differential staining of nuclei after immunosurgery (Fig. 4B and C). Taken together, these results suggested that H2Bub1 is involved in preimplantation development.

Effects of decreased H2Bub1 levels on gene expression in preimplantation embryos

It has been reported that H2Bub1 is involved in developmental regulation at various stages by regulating gene expression in several organisms. Based on the decrease in developmental rate caused by the knockdown of Rnf20, we investigated the effects of decreased H2Bub1 levels on gene expression in preimplantation embryos. Since it was reported that the expression of histone H2A and H2B genes was significantly decreased upon depletion of *RNF20* in human somatic cells [33], we explored the expression of *Hist1h2ab, H2afy, Hist1h2bb, Hist1h2bg* and *Hist2h2bb* in *Rnf20* knockdown

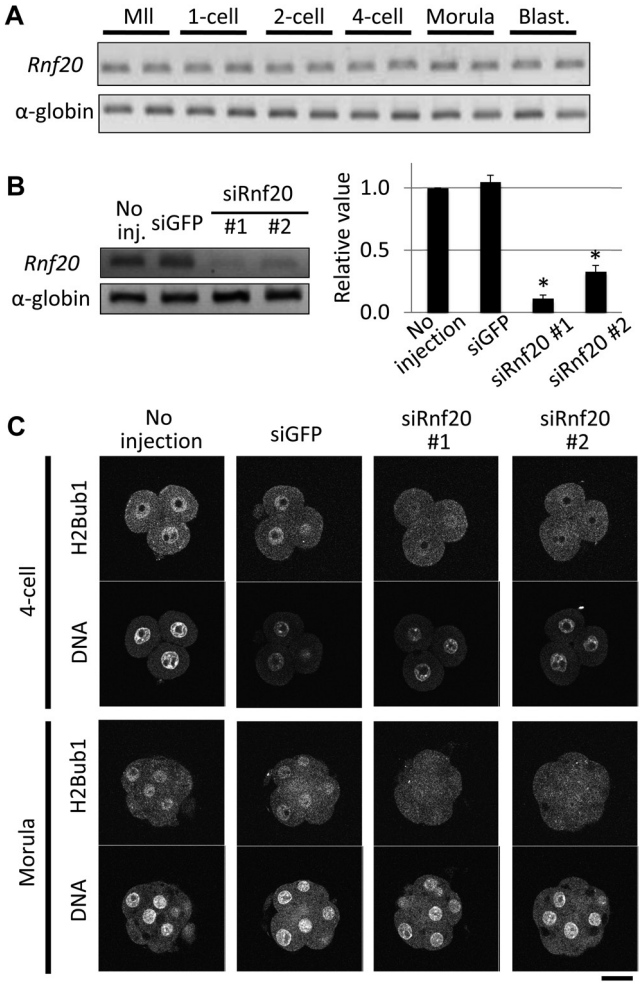

Fig. 2. Knockdown of *Rnf20* by siRNAs decreased H2Bub1 levels in preimplantation embryos. (A) Expression of ring finger protein 20 (*Rnf20)* was examined using RT-PCR in MII stage oocytes (MII) and 1-, 2- and 4-cell, morula and blastocyst (Blast.) stage embryos, which were collected 0, 12, 28, 45, 70 and 96 h after insemination, respectively. Three independent experiments with duplicate samples were performed, and a representative image is shown. Rabbit α-globin was used as an external control. (B) Knockdown of *Rnf20* using siRNAs. Small interfering RNAs against *Rnf20* (siRnf20 #1 and #2) or green fluorescent protein (siGFP) were injected into the cytoplasm of the embryos 2 h after insemination. The *Rnf20* transcript was examined by RT-PCR at the 4-cell stage 45 h after insemination. The intensities of the bands were quantified and shown in the bar graph. The value of no injection was set as 1, and the values of other groups were expressed relative to this value. Asterisks indicate statistically significant difference compared with control siGFP ($P < 0.05$; Student's *t*-test). Four independent experiments were performed. Error bars represent the SEM. (C) Decreases in H2Bub1 in the *Rnf20* knock-down embryos. Embryos injected with siRNAs against *Rnf20* or *GFP* were collected 45 and 70 h after insemination (4-cell and morula stages, respectively) and immunostained with anti-H2Bub1 antibody. DNA was stained with DAPI. Three independent experiments were conducted, and representative images are shown. As a control, embryos without injection and those injected with siGFP were examined.

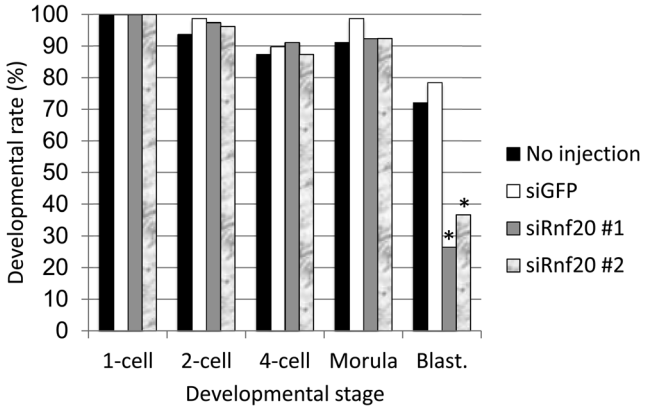

Fig. 3. Effect of *Rnf20* knockdown on the progression of preimplantation development. The small interfering RNAs against *Rnf20* (siRnf20 #1 and #2) or green fluorescent protein (siGFP) were injected into the cytoplasm of the embryos 2 h after insemination. Development to the 1-, 2- and 4-cell, morula and blastocyst (Blast.) stages was observed at 12, 28, 45, 70 and 96 h after insemination, respectively. Four independent experiments were performed, and the data were accumulated. A total of 79 embryos were examined in each experimental group. Black, white, grey and marble bars indicate no injection, siGFP, siRnf20 #1 and siRnf20 #2, respectively. Values indicated by asterisks are significantly lower than the control values (siGFP) ($P < 0.01$; χ^2-test).

embryos. However, no obvious change in the expression of these genes occurred after the H2Bub1 levels decreased in morula stage embryos (Fig. 5).

Discussion

In this study, we examined the role of H2Bub1 in the regulation of preimplantation development. Immunocytochemical analysis showed that H2Bub1 was absent from the pronuclei of early 1-cell stage embryos. However, a weak H2Bub1 signal was detected during the late 1-cell stage. The signal became stronger at the 2-cell stage and remained high until the blastocyst stage (Fig. 1). Knockdown of *Rnf20* via siRNAs decreased H2Bub1 in the nuclei (Fig. 2), which caused a reduction in blastocyst stage development (Figs. 3 and 4). These results suggest that H2Bub1 mediated by RNF20 is involved in the regulation of preimplantation development.

H2Bub1 likely regulates preimplantation development through the regulation of gene expression, since H2Bub1 stimulates transcriptional elongation by RNA polymerase II (RNAPII) [26, 27]. The H2Bub1 levels changed in a manner to the transcriptional activity during preimplantation development. It was previously reported that transcriptional activity was not detected in the early 1-cell stage, appeared in the late stage but at a low level and then increased in the 2-cell stage [21]. The reduction of H2Bub1 by *Rnf20* knockdown via siRNAs caused developmental arrest at the morula stage (Fig. 3), although it was reported that embryos arrested at the 2-cell stage when transcription was inhibited after fertilization [34]. Since the H2Bub1 level was not reduced by siRNAs before the 4-cell stage, the role of H2Bub1 in developmental regulation at the 1- and 2-cell stages remains unknown. It would be interesting to determine whether

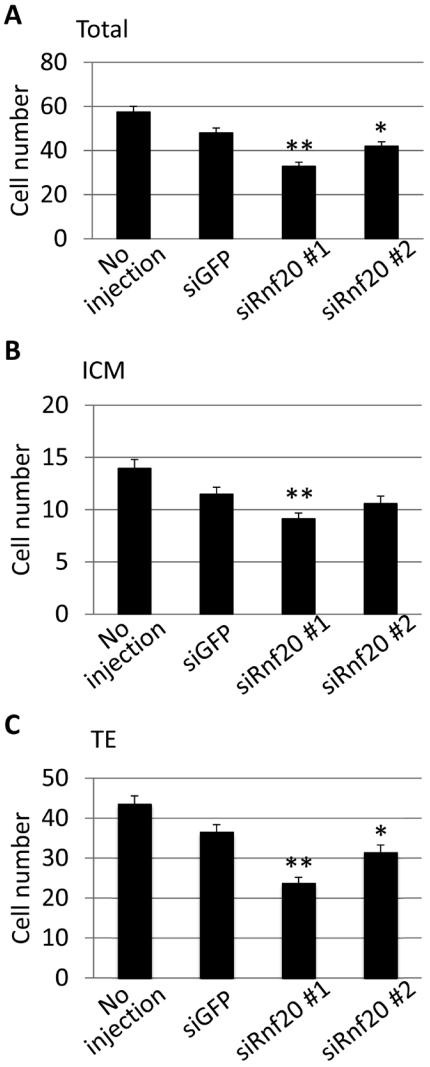

Fig. 4. Effect of *Rnf20* knockdown on the cell numbers in embryos at the blastocyst stage. The embryos were injected with small interfering RNAs against *Rnf20* (siRnf20 #1 and #2) or green fluorescent protein (siGFP) 2 h after insemination and collected at the blastocyst stage 96 h after insemination. The numbers of cells in the whole embryos (A), in the inner cell mass (ICM; B) and in the trophectoderm (TE; C) were determined. For the determination of cell numbers in the ICM and TE, the nuclei of the embryos were stained differentially after immunosurgery. Four independent experiments were performed, and the data were accumulated. In each experimental group, more than 44 embryos were analyzed in total. Error bars indicate standard errors (SE). Values indicated by asterisks are significantly lower than those of the control embryos (siGFP) (based on the Student's *t*-test; *P < 0.05; **P < 0.01).

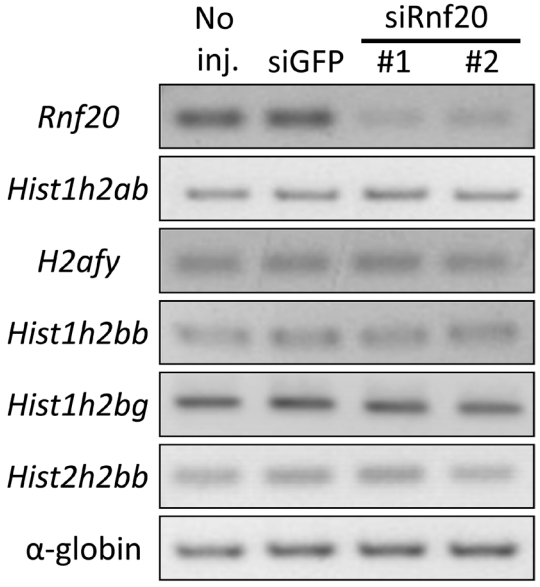

Fig. 5. Effect of *Rnf20* knockdown on the expression of histone H2A and H2B genes. The expression of histone H2A and H2B genes in the *Rnf20* knockdown embryos was examined using RT-PCR. The embryos were injected with siRnf20 #1, siRnf20 #2 or siGFP 2 h after insemination and collected at the morula stage 70 h after insemination. Two independent experiments were performed, and similar results were obtained. A representative image is shown. Rabbit α-globin was used as an external control.

H2Bub1 is involved in zygotic gene activation during this period.

Previous reports suggested that H2Bub1 regulates histone H3K4 and H3K79 methylation, which are tightly associated with active transcription [3, 12, 35–39]. H2Bub1 may regulate changes in gene expression by altering methylation of these residues during preimplantation development. However, H3K79 dimethylation (H3K79me2) is maintained at low levels after fertilization and then increases at the blastocyst stage [40], although H2Bub1 was already detected in the late 1-cell stage and increased in the 2-cell stage in the present study (Fig. 1). The changes in H2Bub1 differed from those in H3K4 trimethylation (H3K4me3). H3K4me3 increased in the male pronuclei at the 1-cell stage. However, it decreased in the 2-cell stage, and the lower level of H3K4me3 was maintained until the blastocyst stage [41, 42]. Thus, H2Bub1 does not seem to act as an upstream mechanism for H3K79me2 and H3K4me3 during preimplantation development. Instead, other mechanisms may cause the dynamic changes in H3K79me2. Indeed, DOT1L (the sole H3K79 methyltransferase) was not localized in the nucleus at the 2-cell stage, and forced nuclear localization of DOT1L caused hypermethylation of H3K79me2 in 2-cell stage embryos [43], suggesting that H3K79me2 is regulated by the nuclear localization of DOT1L during this stage. On the other hand, H3K4me3 is catalyzed by several histone methyltransferases and demethylases [44, 45]. It remains unclear which of these enzymes is involved in the changes in H3K4me3 during preimplantation development.

We analyzed the expression of specific histone genes that are positively regulated by H2Bub1 in human somatic cells in *Rnf20*-deficient preimplantation embryos. No apparent effect on the expression of these genes was observed (Fig. 5). The results suggested that the roles of H2Bub1 in the regulation of gene expression are dependent on cell type. *Rnf20*-deficient ES cells did not show abnormal gene expression profiles, except when treated with retinoic acid to induce differentiation [20]. This may be important for understanding the role of H2Bub1 in preimplantation development and identifying

genes that are misregulated in H2Bub1-deficient preimplantation embryos. A comprehensive analysis of the transcriptome via RNA sequencing may be used to identify these genes.

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