Journal of Reproduction and Development, Vol. 69, No 3, 2023

Original Article

Involvement of linker histone variant H1a in the regulation of early preimplantation development in mice

Satoshi FUNAYA[1)], Yuan WANG[1)], Masataka G. SUZUKI[1)], Masahito IKAWA[2)] and Fugaku AOKI[1)]

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

[2)]*Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan*

Abstract. Linker histone variants regulate higher-order chromatin structure and various cellular processes. It has been suggested that linker histone variant H1a loosens chromatin structure and activates transcription. However, its role in early mouse development remains to be elucidated. We investigated the functions of H1a during preimplantation development using H1a gene-deleted mice. Although H1a homozygous knockout (KO) mice were born without any abnormalities, the number of offspring were reduced when the mothers but not fathers were homozygous KO animals. Maternal H1a KO compromised development during the morula and blastocyst stages, but not differentiation of the inner cell mass or trophectoderm. Thus, maternal linker histone H1a is important in early development.

Key words: Chromatin structure, Linker histone variant, Mouse embryo, Preimplantation development

(**J. Reprod. Dev. 69:** 178–182, 2023)

Linker histones bind to DNA between nucleosomes and are involved in the formation of higher-order chromatin structure [1–3]. Histone variants are expressed by different genes; 11 such variants are known, of which *H1f1*, *H1f2*, *H1f3*, *H1f4*, and *H1f5* (also known as H1a, c, d, e, and b, respectively) are widely expressed in various tissues [4]. The variants are involved in chromatin aggregation [5]. Notably, variant H1a aggregates chromatin more weakly than do H1b, H1c, H1d, and H1e [6]. In human lung fibroblasts, H1b, c, d, and e, the human orthologs of the corresponding mouse variants, are depleted at transcription start sites (TSSs) and enhancers of actively transcribed genes, but significant amounts of H1a remain [7]. H1b, c, d, and e are associated with repressed chromatin that is also associated with HP1, whereas H1a tends to be abundant in regions of active chromatin [7], suggesting that H1a may loosen chromatin structure and activate transcription. However, the roles played by H1a in early development remain to be elucidated. We previously used RNA-seq to analyze the expression levels of linker histone variants, and found that H1a was the most highly expressed variant at the one- and two-cell stages [8]. By contrast, H1b, c, d, and e were expressed at low levels at these stages.

The epigenetic pattern and chromatin structure regulate changes in the dynamic gene expression pattern during preimplantation development [9, 10]. It is possible that H1a plays an important role in this process. The development of H1a knockout (KO) mice has been investigated by other research groups; H1a homozygous KO did not adversely affect terms of survival, and fertility [11, 12]. However, neither the litter sizes of H1a KO mice nor the effect of H1a KO on early development has been studied. We thus generated H1a KO mice and examined the role played by H1a in preimplantation development. Female H1a homozygous KO mice evidenced reduced litter sizes. Furthermore, the rate of development to the blastocyst stage was reduced in embryos of maternal H1a KO mice, although differentiation into blastocyst inner cell mass (ICM) and trophectoderm (TE) cells was normal.

Received: February 14, 2023
Accepted: April 4, 2023
Advanced Epub: April 17, 2023

Correspondence: F Aoki (e-mail: aokif@edu.k.u-tokyo.ac.jp), S Funaya (e-mail: funaya@edu.k.u-tokyo.ac.jp)

Materials and Methods

Animals

The mice were bred in-house or purchased from Japan SLC (Shizuoka, Japan). All animal procedures were 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.

Generation of H1a KO mice using the CRISPR/Cas9 system

H1a KO C57BL/6J mice were generated using the CRISPR/Cas9 system. gRNA specific for the H1a gene and its flanking regions was used to delete a part of the H1a gene. The method of CRISPR/Cas9 has previously been described [13]. To confirm H1a KO, primers binding to regions outside of the H1a gene were used for PCR of extracted genomic DNA using the KOD FX Neo system (Toyobo, Osaka, Japan; catalog no. KFX-201). The amplified products were electrophoresed and the bands identified the genotypes. The gRNA target sequences (all 5′ to 3′) were gRNA1 GGAGACGGCGCCAGTCGCTC, gRNA2: TGGAGAGGGTAGCGCCTCCC and the primer sequences for PCR were ACTGAAACCCGCAGAGAAGG (forward) and GTGAACATTGCAGTACATGG (reverse). PCR featured denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min, followed by a final extension at 72°C for 3 min.

Mating

Sexually mature male and female mice (more than 8 weeks of age) of each genotype were mated and the number of pups per litter were counted.

In vitro fertilization

Three- to four-week-old female mice were subjected to *in vitro* fertilization. The mice were injected with 5 IU pregnant mare serum gonadotropin (PMSG; ASKA Pharmaceutical, Tokyo, Japan) followed by 7.5 IU human chorionic gonadotropin (hCG; ASKA Pharmaceutical) 48 h after PMSG injection. Mll-stage oocytes were collected from the ampullae of oviducts 16 h after hCG injection. Sperm were collected from the cauda epididymis of sexually mature mice and cultured in human tubal fluid (HTF) medium [14] for 2 h. *In vitro* fertilization was performed by adding sperm to HTF medium containing Mll-stage oocytes. After 6–9 h, embryos were washed several times with K+-modified simplex optimized medium (KSOM) [15] medium using a glass pipette to remove cumulus cells. Only embryos with two pronuclei evident under a stereomicroscope were selected.

Immunostaining

To detect H1FOO, NANOG, and CDX2, embryos were incubated with PBS containing 3.7% (w/v) PFA (Wako, Osaka, Japan; catalog no. 160-16061) and 0.2% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA; catalog no. T9284) for 20 min at room temperature. After fixation, the embryos were washed several times with PBS with 1% (w/v) BSA (Sigma-Aldrich; catalog no. A9647) and incubated with anti-H1foo rabbit antibody (1:500 dilution; prepared in-house as described previously [16]), anti-Cdx2 mouse antibody (1:500 dilution; BioGenex, San Ramon, CA, USA; catalog no. MU392A-UC), and anti-Nanog rabbit antibody (1:500 dilution; Abcam, Cambridge, UK; catalog no. ab80892) diluted in PBS with 1% (w/v) BSA and 0.2% (v/v) Tween (MP Biomedicals, Santa Ana, CA, USA; catalog no. 103168) overnight at 4°C. The embryos were washed several times with PBS with 1% (w/v) BSA and incubated with secondary antibody (1:100 dilution; Thermo Fisher Scientific, Waltham, MA, USA, A10042, A11001) in PBS with 1% (w/v) BSA and 0.2% (v/v) Tween for 1 h at room temperature. The embryos were washed with PBS with 1% (w/v) BSA and mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, CA, USA; catalog no. H1200). The samples were observed under an Olympus FV3000 laser scanning microscope (Olympus, Tokyo, Japan). To evaluate NANOG and CDX2 status, embryo images were Z-stacked and the numbers of ICM and TE cells counted. We took 28–47 pictures for the z-stack image. The blastomeres stained with only Cdx2 or Nanog were judged as TE or ICM cells, respectively. There were also some blastomeres stained with both of Cdx2 and Nanog. They were judged as TE cells, since the intensity of fluorescence of Nanog was weaker in these cells than that of cells stained with only Nanog.

Symbols and gene names of linker histone variants

The symbols and synonyms of the linker histone variants are *H1f1*/H1a, *H1f5*/H1b, *H1f2*/H1c, *H1f3*/H1d, *H1f4*/H1e, and *H1f8*/H1foo.

Results

Generation of H1a KO mice using CRISPR/Cas9

We generated H1a KO mice using the CRISPR/Cas9 system (Fig. 1A). Deletion of a large part of H1a was confirmed by PCR (Fig. 1B).

Effect of H1a KO on mouse fertility

To examine whether H1a homozygous mice were born normally, we mated male and female H1a heterozygous mice. The genotype ratios of offspring from the heterozygous intercrosses followed Mendelian laws (Fig. 2A). There were no differences in body weight

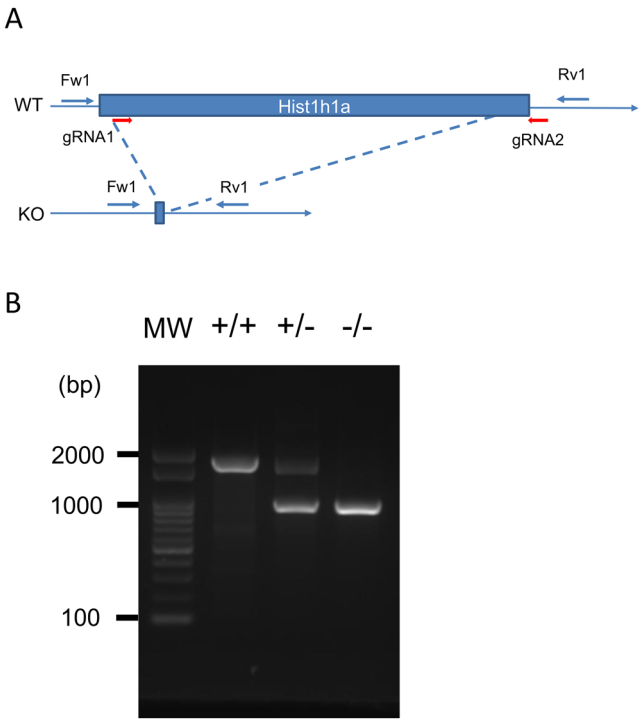

Fig. 1. Generation of H1a KO mice. (A) Schematic of the genomic structures of WT and KO mice. Red arrows indicate the gRNA locations. Blue arrows indicate the positions of primers used to confirm KO. The size of H1a gene is 745 bp. (B) Genotypic verification of H1a KO mice via genomic PCR. +/+, +/–, and –/– indicate the wild-type (WT), heterozygous KO, and homozygous KO genotypes, respectively.

21 days after birth among wild-type, heterozygous, or homozygous mice (Fig. 2B). These results are consistent with previous studies indicating that H1a homozygous KO mice are born normally [11, 12].

Next, we investigated the effect of H1a KO on male and female fertility. The litter sizes on crossbreeding of H1a homozygous KO males and wild-type females and wild-type males and females were similar. However, the litter sizes fell when H1a homozygous KO females were mated with wild-type or homozygous males (Fig. 2C), suggesting that H1a contributed to female mouse fertility.

Effect of H1a KO on preimplantation development

We investigated the effect of maternal H1a KO on preimplantation development. MII- stage oocytes obtained from H1a-null female mice were fertilized with spermatozoa from wild-type males. Maternal H1a KO had a detrimental effect on preimplantation development, especially from the morula to blastocyst stage (Fig. 3A); thus, it was possible that H1a KO might affect differentiation of the ICM and TE. Hence, we immunostained embryos for NANOG and CDX2 (markers of the ICM and TE, respectively). The numbers of stained cells did not differ from those of wild-type embryos (Figs. 3A, B), indicating that maternal H1a is important in terms of preimplantation development but does not affect differentiation into the ICM and TE.

Effect of H1a KO on the deposition of other linker histone variants

Eleven linker histone variants have been identified in mice. H1a is highly expressed at the one- and two-cell stages [8]. To determine whether the absence of H1a affects the localization of other linker

A

Number of analyzed offspring	Genotype ratio of pups (%)		
	(+/+)	(+/-)	(-/-)
78	28.2	46.2	25.6

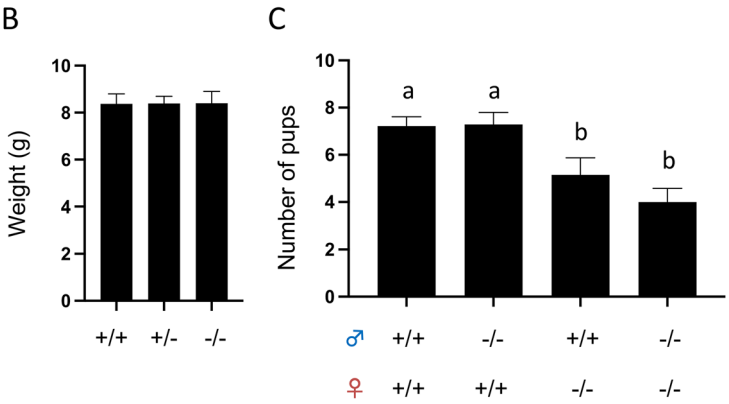

Fig. 2. Effects of H1a KO on the fertilities of male and female mice. (A) Genotype ratios of offspring derived from crosses of heterozygous KO mice. (B) Average body weights at 21 days after birth. The symbols +/+, +/–, and –/– indicate the wild-type (WT), heterozygous KO, and homozygous KO genotypes, respectively. Error bars indicate the SEs. (C) Litter size on crossing of H1a paternal and/or maternal KO mice. ♂ and ♀ indicate the paternal and maternal alleles, respectively. Different characters indicate significant differences between the groups ($P < 0.05$, one-way ANOVA with the Tukey multi-comparisons test).

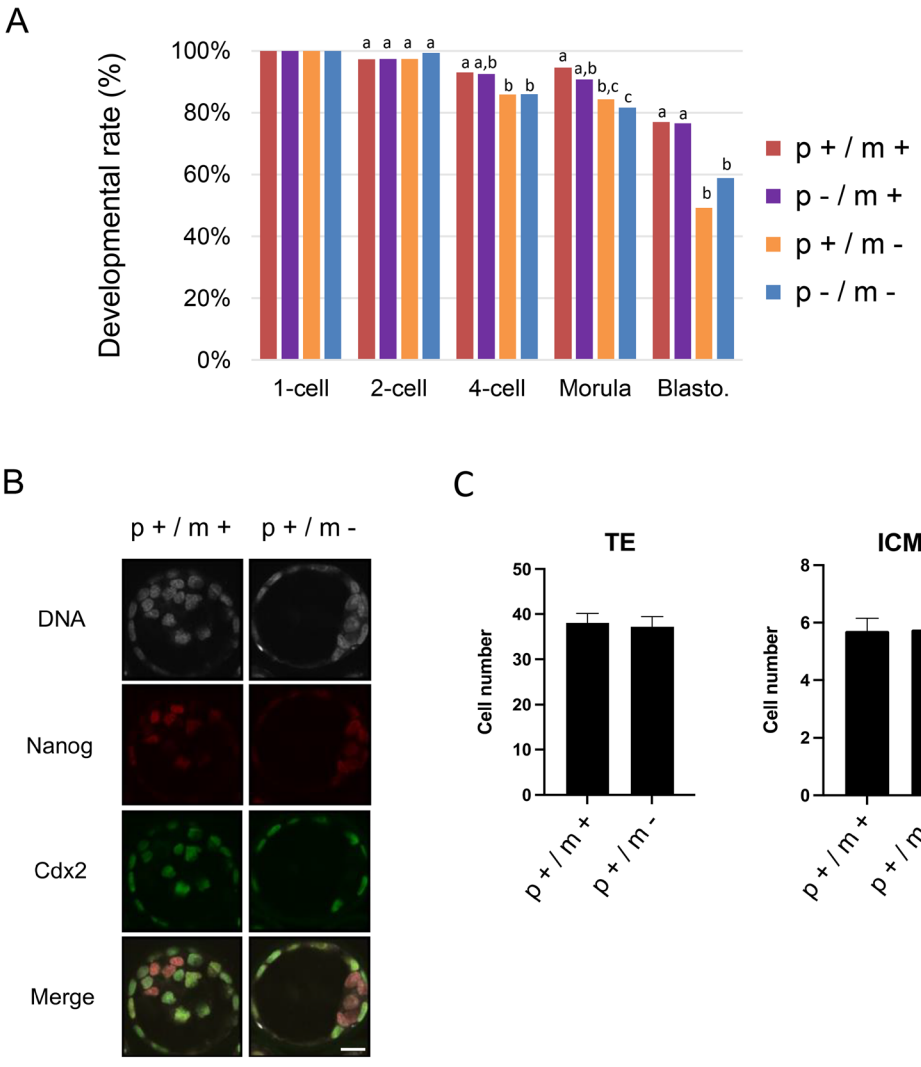

Fig. 3. Effect of H1a KO on preimplantation development. (A) The developmental rates of embryos generated on fertilization of oocytes and spermatozoa from H1a KO and wild-type (WT) mice. P and m indicate paternal and maternal alleles, respectively; + and – represent WT and KO, respectively. More than 120 embryos were analyzed. Zygotes were observed at 12 h post-insemination (hpi) and then at 28, 48, 72, and 96 hpi, at which times most WT embryos had attained the 2- and 4-cell, and the morula and blastocyst stages, respectively. No common characters among groups indicates a significant difference. ($P < 0.05$; χ^2 or Fisher's exact test). (B) Immunofluorescence images of NANOG and CDX2 of WT and maternal H1a KO blastocysts at 96–102 hpi. Scale bar = 20 µm. (C) The numbers of ICM and TE cells in WT and maternal H1a KO blastocysts. Over 21 embryos were analyzed. Error bars indicate the SEs.

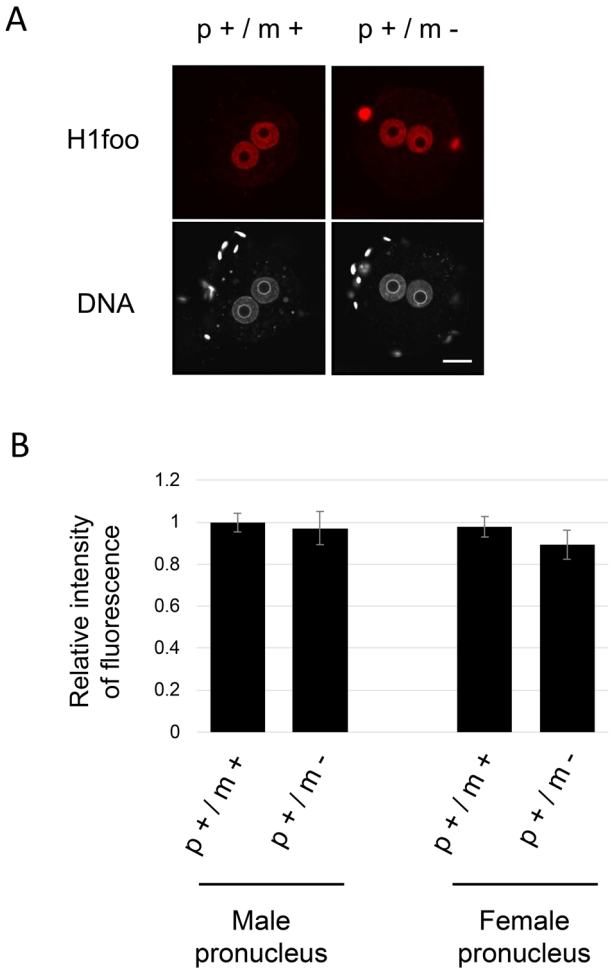

Fig. 4. Effect of H1a KO on H1foo deposition in one-cell embryos. (A) Immunofluorescence images of H1foo in WT and maternal H1a KO one-cell embryos at 8–10 h post insemination (hpi). P and m indicate paternal and maternal alleles, respectively. + and – indicate WT and KO, respectively. Scale bar = 20 µm. (B) Quantification of the H1FOO fluorescence signals from WT and maternal H1a KO one-cell embryos. The H1foo signal intensities were corrected by reference to those of DAPI. The average male pronuclear value of WT embryos (p+/m+) was set to 1 and then relative values were calculated. At least three independent experiments were performed using each experimental group, and more than 18 embryos in total were analyzed. Error bars indicate the SEs.

histone variants at the one-cell stage, we performed immunostaining of the linker histone variant H1f8 (also known as H1foo) that is highly expressed at the 1-cell stage [8, 17]. The signal intensity of H1foo was similar in maternal H1a KO and wild-type one-cell embryos (Figs. 4A, B), suggesting that loss of H1a does not affect the localization of H1foo in one-cell stage embryos.

Discussion

We examined the role of H1a in early embryonic development. The litter size and our evaluation of preimplantation development in H1a KO mice revealed that maternal H1a was important for early development. In early preimplantation embryos, both H1a and H1foo seem to play important regulatory roles; these are the most strongly expressed of all 11 linker histone variants [8, 17]. However, H1foo KO male and female mice were normally fertile [18]. We previously showed that H1foo KD embryos developed normally to the blastocyst stage [8]. In contrast to H1foo, the developmental rate to the blastocyst stage was reduced in embryos of H1a KO mothers, indicating that H1a plays a more important role in female fertility and early embryo development than does H1foo. The H1foo proteins of humans and mice show 40% similarity but the H1a proteins show 80% similarity. The much higher sequence conservation of H1a suggests that H1a plays the more important role in formation of the chromatin structure that affects developmental regulation [4].

Maternal H1a KO compromised development from the morula to the blastocyst, but not differentiation into the ICM and TE. The total amount of somatic linker histones increased when core histone variant H3.3 was knocked down in preimplantation embryos; this triggered chromosome mis-segregation and developmental arrest at the morula stage [19]. Although we could not immunostain for the somatic linker histones H1b, c, d, or e because antibodies are not available, it is possible that nuclear deposition of certain H1b-e variants may be increased after H1a KO. As such variants condense chromatin more strongly that does H1a, maternal H1a KO may trigger formation of condensed chromatin and thus chromosome mis-segregation in early embryos.

Although H1foo is highly expressed in one-cell embryos, as is H1a, H1foo deposition was not affected by H1a deletion. The reason is unclear, but it is possible that the genomic locations of H1a and H1foo differ and that either protein cannot bind to sites preferred by the other. One comprehensive study revealed that H1a appeared to bind to genomic locations not bound by H1b, c, d, or e [7]. A comprehensive analysis of histone genomic distribution in early embryos is required, using antibodies specific for H1a and H1foo.

Conflict of interests: The authors have nothing to declare.

Acknowledgments

This study was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (nos. 19H05752, 21H04752, 22K15022, and 16H06276).

References

1. **Thoma F, Koller T, Klug A.** Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. *J Cell Biol* 1979; **83**: 403–427. [Medline] [CrossRef]
2. **Wolffe AP.** Histone H1. *Int J Biochem Cell Biol* 1997; **29**: 1463–1466. [Medline] [CrossRef]
3. **Bednar J, Horowitz RA, Grigoryev SA, Carruthers LM, Hansen JC, Koster AJ, Woodcock CL.** Nucleosomes, linker DNA, and linker histone form a unique structural motif that directs the higher-order folding and compaction of chromatin. *Proc Natl Acad Sci USA* 1998; **95**: 14173–14178. [Medline] [CrossRef]
4. **Pan C, Fan Y.** Role of H1 linker histones in mammalian development and stem cell differentiation. *Biochim Biophys Acta* 2016; **1859**: 496–509. [Medline] [CrossRef]
5. **Clausell J, Happel N, Hale TK, Doenecke D, Beato M.** Histone H1 subtypes differentially modulate chromatin condensation without preventing ATP-dependent remodeling by SWI/SNF or NURF. *PLoS One* 2009; **4**: e0007243. [Medline] [CrossRef]
6. **Khadake JR, Rao MR.** DNA- and chromatin-condensing properties of rat testes H1a and H1t compared to those of rat liver H1bdec; H1t is a poor condenser of chromatin. *Biochemistry* 1995; **34**: 15792–15801. [Medline] [CrossRef]
7. **Izzo A, Kamieniarz-Gdula K, Ramírez F, Noureen N, Kind J, Manke T, van Steensel B, Schneider R.** The genomic landscape of the somatic linker histone subtypes H1.1 to H1.5 in human cells. *Cell Rep* 2013; **3**: 2142–2154. [Medline] [CrossRef]
8. **Funaya S, Ooga M, Suzuki MG, Aoki F.** Linker histone H1FOO regulates the chromatin structure in mouse zygotes. *FEBS Lett* 2018; **592**: 2414–2424. [Medline] [CrossRef]
9. **Du Z, Zhang K, Xie W.** Epigenetic reprogramming in early animal development. *Cold Spring Harb Perspect Biol* 2022; **14**: 14. [Medline] [CrossRef]
10. **Aoki F.** Zygotic gene activation in mice: profile and regulation. *J Reprod Dev* 2022; **68**: 79–84. [Medline] [CrossRef]

11. **Rabini S, Franke K, Saftig P, Bode C, Doenecke D, Drabent B.** Spermatogenesis in mice is not affected by histone H1.1 deficiency. *Exp Cell Res* 2000; **255**: 114–124. [Medline] [CrossRef]
12. **Lin Q, Inselman A, Han X, Xu H, Zhang W, Handel MA, Skoultchi AI.** Reductions in linker histone levels are tolerated in developing spermatocytes but cause changes in specific gene expression. *J Biol Chem* 2004; **279**: 23525–23535. [Medline] [CrossRef]
13. **Abbasi F, Miyata H, Shimada K, Morohoshi A, Nozawa K, Matsumura T, Xu Z, Pratiwi P, Ikawa M.** RSPH6A is required for sperm flagellum formation and male fertility in mice. *J Cell Sci* 2018; **131**: 131. [Medline]
14. **Quinn P, Begley AJ.** Effect of human seminal plasma and mouse accessory gland extracts on mouse fertilization in vitro. *Aust J Biol Sci* 1984; **37**: 147–152. [Medline] [CrossRef]
15. **Lawitts JA, Biggers JD.** Culture of preimplantation embryos. *Methods Enzymol* 1993; **225**: 153–164. [Medline] [CrossRef]
16. **Funaya S, Kawabata Y, Sugie K, Abe KI, Suzuki Y, Suzuki MG, Aoki F.** Involvement of the linker histone H1Foo in the regulation of oogenesis. *Reproduction* 2022; **164**: 19–29. [Medline]
17. **Tanaka M, Hennebold JD, Macfarlane J, Adashi EY.** A mammalian oocyte-specific linker histone gene H1oo: homology with the genes for the oocyte-specific cleavage stage histone (cs-H1) of sea urchin and the B4/H1M histone of the frog. *Development* 2001; **128**: 655–664. [Medline] [CrossRef]
18. **Sánchez-Sáez F, Sainz-Urruela R, Felipe-Medina N, Condezo YB, Sánchez-Martín M, Llano E, Pendás AM.** The oocyte-specific linker histone H1FOO is not essential for mouse oogenesis and fertility. *Cells* 2022; **11**: 11. [Medline] [CrossRef]
19. **Lin CJ, Conti M, Ramalho-Santos M.** Histone variant H3.3 maintains a decondensed chromatin state essential for mouse preimplantation development. *Development* 2013; **140**: 3624–3634. [Medline] [CrossRef]