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Original Article

Cleavage-embryo genes and transposable elements are regulated by histone variant H2A.X

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Abstract. During mammalian preimplantation development, stimulation of zygotic genome activation (ZGA) and transposable elements (TEs) shapes totipotency profiling. A rare mouse embryonic stem cells (mESCs) subpopulation is capable of transiently entering a state resembling 2-cell stage embryos, with subtypes of TEs expressed and ZGA genes transiently activated. In this study, we found that deletion of H2A.X in mESCs led to a significant upregulation of ZGA genes and misregulated TEs. ChIP-seq analysis indicated a direct association of H2A.X at the *Dux* locus for silencing the *Dux* gene and its downstream ZGA genes in mESCs. We also demonstrated that histone variant H2A.X is highly enriched in human cleavage embryos when ZGA genes and TEs are active. Therefore, we propose that H2A.X plays an important role in regulating ZGA genes and TEs to establish totipotency.

Key words: *Dux*, Histone variant, H2A.X, Transposable element, Zygotic genome activation

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Upon fertilization, chromatin reorganization occurs sequentially during preimplantation development [1]. During this period, gametes undergo epigenetic reprogramming to revert to a totipotent state by initiating zygotic gene activation (ZGA) and stimulation of transposable elements (TEs) [2, 3]. Major ZGA peaks at the 2-cell stage in mice, but occur in the 4–8 cell embryo stage in humans. As one of the most critical developmental events, ZGA is generally characterized by the activation of ZGA genes, such as *Zscan4*, *Dux*, and the endogenous retrovirus ERVL. ERVL is a downstream target of *Dux* and a hallmark of ZGA. Besides ERVL, other TEs, such as LINE and SINE, are also enriched in early embryos, especially subtypes of young TEs. Mouse embryonic stem cells (mESCs) are isolated from the inner cell mass (ICM) of developing blastocysts and are generally in a primed pluripotent state. The chromatin status of mESCs is similar to that of early embryos, and chromatin structure is more open in mESCs than in somatic cells, making mESCs a good model for studying the regulation of TEs. Interestingly, a rare population of mESCs was also reported to transiently express ZGA transcripts when it sporadically transitions into an early embryonic-like state [4]. Therefore, mESCs were also used to identify ZGA activators and repressors [5].

Histones are fundamental structural components of chromatin in cells. In addition to canonical histones H2A, H2B, H3, and H4, histone variants and their modifications are also typically involved in the specific regulation of chromatin events by expanding the dynamics of nucleosomes and even driving diverse cell fate decisions [6, 7]. Moreover, some histone variants are species specific [8]. Different histone variants have distinct positioning and dynamics in cells, which are assembled into nucleosomes by different molecular chaperones and interact with various chromatin remodeling complexes, thus replacing canonical histones or substitution among various variants during development and cell differentiation [9]. Structural differences introduced by a core histone variant can impact interactions among histones, leading to the transformation of nucleosome stability and chromatin opening or compaction [10]. H2A.Z is a long-standing H2A variant that is highly conserved among species. Deletion of H2A.Z has been reported to be lethal in Drosophila, Tetrahymena, and mice [11–13]. Additionally, H2A.Z has been shown to be involved in transcriptional activation and epigenetic memory [14, 15]. Another H2A variant, macroH2A, is a vertebrate-specific histone variant that contains an N-terminal H2A-like region (65% identity to H2A) and a large C-terminal non-histone region [16]. The macroH2A variant is typically associated with condensed chromatin at the inactive X (Xi) chromosome [17], and has been shown to block reprogramming activity [18, 19], indicating its role in transcriptional repression. H2A.X was one of the earliest reported H2A variants, and its major function is regarded as a DNA damage sensor[20]. In mammals, H2A.X shares sequence similarities of up to 95% of canonical H2A [10] and is highly conserved among species [21]. Notably, H2A.X contains a unique SQ motif at its C-terminus and is invariant in species with regard to its sequence and position relative to its C-terminus [22]. Upon DNA damage or induced DNA double-strand breaks (DSBs), the serine in the SQ motif of H2A.X becomes phosphorylated by DNA-dependent protein kinases (ATR/ATM), forming γH2A.X foci to recruit DNA repair complexes [23, 24]. Recent work has shown that H2A.X regulates CDX2 and its targeted extraembryonic genes, and determines the developmental potential of stem cells, indicating regulatory roles of H2A.X in the cell fate control-related transcriptional network [25]. However, the functions of H2A.X beyond DNA repair remain largely unexplored.

In this study, we aimed to identify the potential functions and deposition of H2A variant H2A.X using stem cell models and human

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early embryos, and demonstrated that H2A.X regulated TEs and repressed ZGA gene expression through deposition at *Dux* gene loci for inhibition. Additionally, we showed that H2A.X was abundantly deposited in human cleavage embryos when ZGA genes and TEs were active. Our results support the understanding of the important role that H2A.X plays in modulating the expression of ZGA genes and TEs.

Materials and Methods

RNA-seq dataset analysis

Raw reads were processed with Cutadapt v1.16 to remove adapters and perform quality trimming with default parameters except for quality cutoff and minimum length, both of which were equal to 20. Trimmed reads were mapped to the mouse genome (GENCODE release M23) using STAR with default settings. RSEM was used to calculate the FPKM values, and a pseudo count of one was added to the FPKM value for each gene. The TE transcripts program [26] was used to obtain counts for transposable elements with default parameters. Read counts of gene and TE transcripts were normalized to the total aligned counts. Dot-and-box plots and heatmaps were generated by R. RNA-seq results for processing included H2A.X knockout mESCs (GSE49147) and ERVL-positive/negative mESCs (GSE33923). The ZGA gene list has been previously reported [27]. The RNA-seq dataset of the mESCs transcriptome before and after *Dux* overexpression was also used [28].

ChIP-seq analysis

Raw reads were processed with Cutadapt v1.8.1, to remove adapters and perform quality trimming. Trimmed reads were mapped to the UCSC mm10 assembly using Bowtie2 with default parameters. An Integrative Genomics Viewer (Broad Institute and the Regents of the University of California, USA) was used for visualization. The reported H2A.X ChIP-seq result in mESCs was from GSE42309.

Collection of early human embryos for immunostaining

Human preimplantation embryos were obtained from the Reproductive Medicine Center, Tongji Medical College, HUST. A total of 29 frozen-thawed embryos abandoned by the patients were included in this study. Ethical approval for the study was obtained from the CEIC (Ethics Committee for Clinical Research) of the Reproductive Medicine Center, Tongji Medical College, HUST. All people included in the study gave informed consent.

Embryos were fixed and permeabilized with 3.7% paraformaldehyde and 0.5% Triton (Sigma, St. Louis, MO, USA) in PBS for 45 min. Embryos were then washed three times in PBS-T (0.05% Tween in PBS), blocked for 30 min, and incubated with primary antibodies overnight at 4°C, followed by three washes in PBS-T and incubation for 2 h at room temperature (25°C) with secondary antibodies and 20 μg/ml Hoechst. The primary antibodies used included anti-H2A.X (ab11175, Abcam, Cambridge, UK) and anti-H2A.Z (ab4174, Abcam), anti-macroH2A (61428, active motif, Carlsbad, CA, USA), and anti-H2A (D210-3, MBL, Nagoya, Japan). Confocal microscopy was performed using a laser scanning microscope (LSM780, Zeiss, Oberkochen, Baden-Württemberg, Germany) at Tongji Medical College, HUST.

Statistical analysis

The Wilcoxon rank sum test with continuity correction was used to calculate P values.

Results

mESCs were reported to transiently activate ZGA genes to show totipotency and other features of 2-cell embryos, and therefore have been widely used to study epigenetic regulation of ZGA events [4]. mESCs also express young TEs because of their relatively open chromatin structure, and the population of mESCs with activated ZGA genes has higher chromatin accessibility and specific activation of ERVL and other TEs [27]. Therefore, to explore the role of H2A.X in regulating the expression of ZGA genes and TEs, we examined the transcriptome of mESCs (derived from 129sv mice) before and after H2A.X depletion [25]. First, we analyzed the expression of ZGA genes in control and H2A.X knockout mESCs and found that their overall expression was enhanced after H2A.X depletion, and the ZGA gene list we used was also proven to be enriched in a totipotent mESCs population marked by ERVL promoter activity [4] (Fig. 1A). Notably, some representative ZGA genes were significantly upregulated after H2A.X knockout, including *Zfp352*, *Sp110*, *Tcstv3*, and *Zscan4* gene families (Fig. 1B). We then examined the expression of representative maternal factors and pluripotent factors, but found that none of them showed obvious changes in expression (Fig. 1C).

The expression of ZGA genes is regulated by activators and repressors in mESCs. To identify how ZGA genes were activated in the absence of H2A.X in mESCs, we examined the expression of reported ZGA regulators in the transcriptome of mESCs before and after H2A.X depletion (Fig. 2A). We found that in H2A.X knockout mESCs, only the two ZGA activators, *Dux* and *Zscan4*, were upregulated. As a master activator of ZGA genes, *Dux* can stimulate ZGA genes, including *Zscan4* family genes [29–31], and *Zscan4* also upregulates the *Dux* gene [32, 33]. To identify whether H2A.X is directly associated with genomic loci of ZGA activators, we analyzed ChIP-seq results of H2A.X in mESCs. Interestingly, we observed a signal of H2A.X occupancy at the *Dux* locus, instead of the *Zscan4* genes (Fig. 2B). This result showed that ZGA genes were repressed by H2A.X-mediated *Dux* downregulation via direct incorporation of H2A.X at the *Dux* locus. To identify whether *Dux* upregulation significantly contributed to H2A.X-mediated repression of ZGA genes, the fold change of ZGA gene expression was compared between the H2A.X knockout group and the *Dux* overexpression group (Fig. 2C). We noticed that genes with higher upregulation induced by *Dux* had increased expression in the absence of H2A.X, supporting the critical role of *Dux* in H2A.X-mediated ZGA gene silencing. We also examined the expression of TEs before and after H2A.X knockout (Fig. 2D). As expected, ERVL, a direct target of *Dux*, was upregulated in mESCs deficient in H2A.X. Moreover, ERVK expression was downregulated when H2A.X was depleted.

To understand how H2A.X and other H2A variants are involved in totipotency establishment, it is important to investigate their deposition in cleavage embryos expressing ZGA genes and blastocysts with silenced ZGA genes and activated pluripotency networks. Deposition of H2A variants in mouse early embryos was previously identified [34], with nuclear deposition of histone H2A variants changed during preimplantation development. Generally, all of the H2A variants were abundantly incorporated and deposited into chromatin in oocytes, but only H2A.X was significantly incorporated into 1–2-cell stage embryos, and macroH2A, H2A.Z, and H2A were enriched in the chromatin of blastocysts. In this study, we collected human cleavage-stage embryos (Fig. 3A, Supplementary Fig. 1) and blastocysts (Fig. 3B) to examine the deposition of H2A variants by immunofluorescence using specific antibodies. We found that H2A.X, canonical H2A, and H2A.Z were deposited in both human cleavage

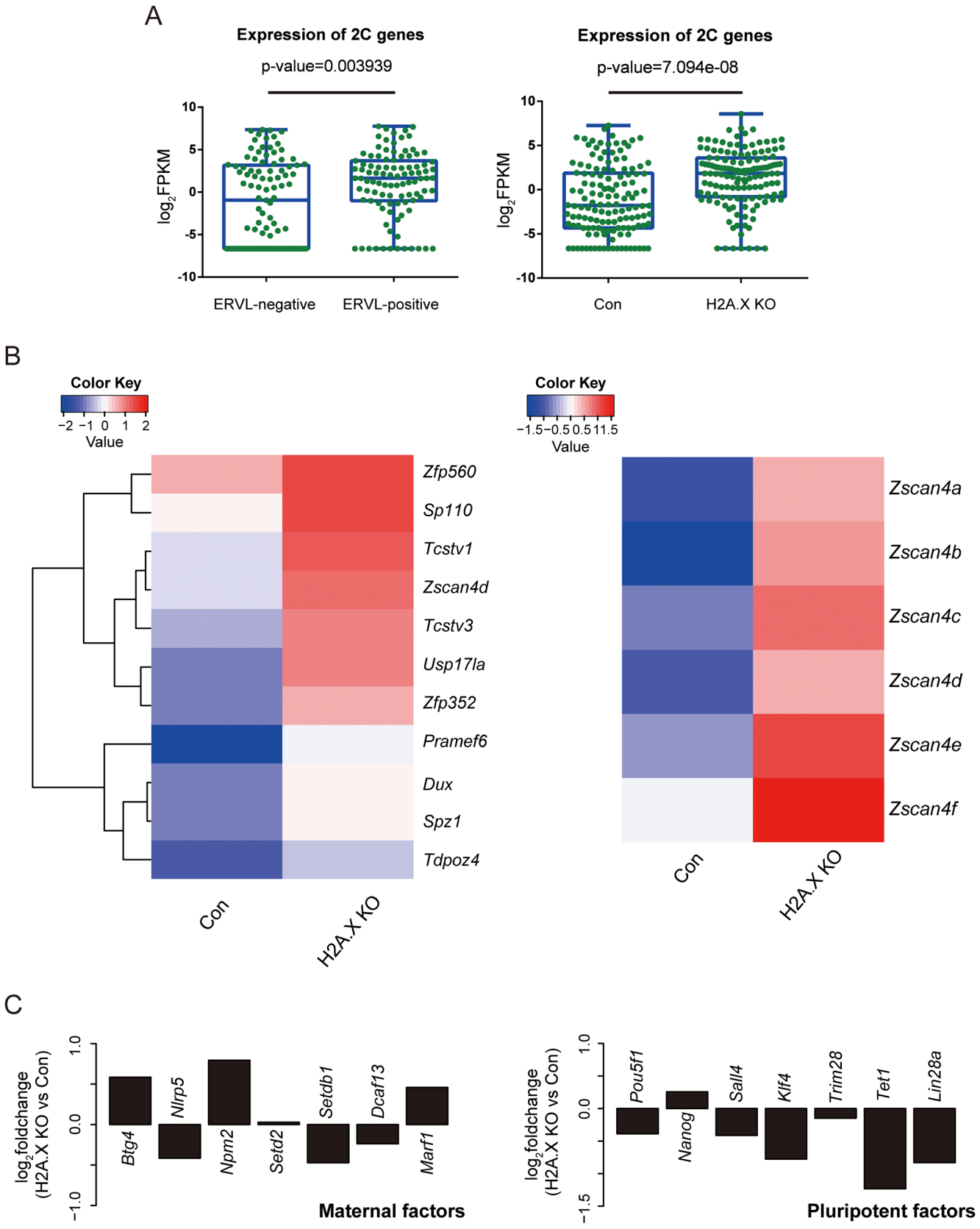

Fig. 1. Depletion of H2A.X led to misregulation of ZGA genes in mESCs. A: Dot-and-box plot shows higher expression (log_2FPKM) of ZGA genes in ERVL-positive mESCs than ERVL-negative mESCs. Similarly, expression of ZGA genes was higher in H2A.X KO mESCs than the control. Displayed using a dot-and-box plot. B: Heatmap of abundance (log_2FPKM) of representative ZGA genes in H2A.X KO mESCs and control. Expression of *Zscan4* family genes in two groups is also shown by heatmap. C: Expression changes (log_2Fold change of FPKM) of maternal and pluripotent factors in H2A.X KO versus control mESCs.

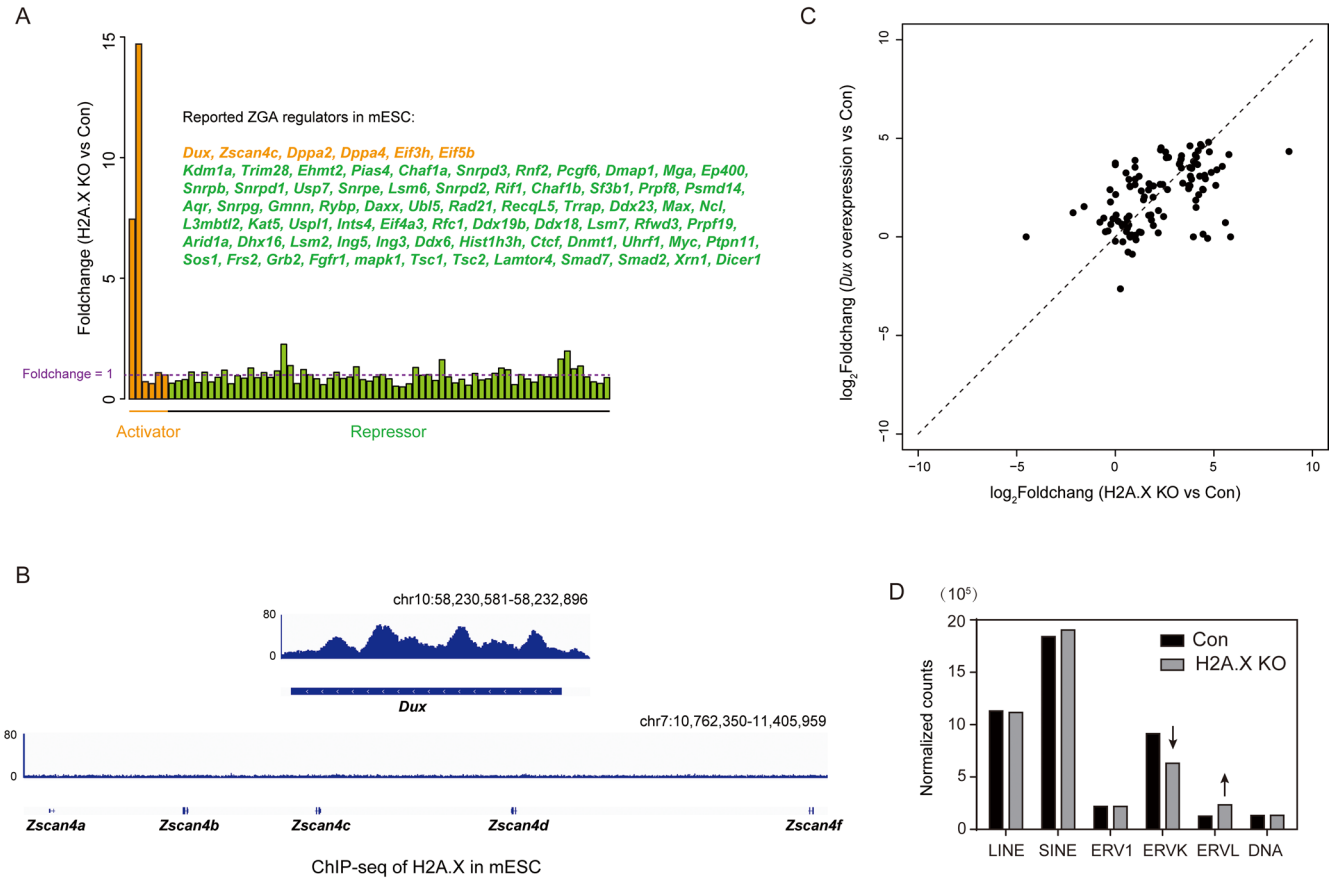

Fig. 2. H2A.X regulates ZGA genes and TEs in mESCs. A: Fold change in expression (FPKM) of reported ZGA regulators in H2A.X KO versus control mESCs. Orange and aqua indicate the ZGA gene activator and repressor, respectively. B: Integrative Genomics Viewer (IGV) visualization of H2A.X occupancy at the *Dux* locus and *Zscan4* family gene locus in mESCs. C: Scatterplot showing the correlation of enhanced ZGA gene expression upon *Dux* overexpression versus H2A.X KO in mESCs. D: Abundance of subfamilies of TEs in control and H2A.X KO mESCs. Note that ERVK and ERVL were misregulated in the absence of H2A.X.

stage (8-to-16-cell) embryos and blastocysts. However, macroH2A was detectable only in the blastocyst stage. Although there are differences in the deposition of H2A variants between human and mouse embryos, H2A.X in H2A variants was consistently enriched during the ZGA period in both human and mouse embryos.

Discussion

Stimulation of ZGA genes is required for the establishment of totipotency and occurs during cellular reprogramming. Currently, the most famous factor for direct ZGA stimulation is the transcription factor *Dux*. *Dux* is activated during the ZGA process with unclear mechanisms and can improve reprogramming efficiency [35]. Although *Dux* deficiency during development is not lethal [36], insufficient ZGA and reduced survivability of embryos have been reported [37]. One possible reason may be that more *Dux* paralogs exist in the mouse genome [38]. Another intriguing event accompanied by ZGA is the activation of the TEs. TEs are enriched in early embryos and is involved in complex regulatory networks, including controlling the timing of ZGA [39, 40]. Despite extensive studies on the regulation of ZGA and TEs, important regulatory factors and mechanisms remain largely unexplored. In the current study, we used mESCs as a cellular model to identify if H2A.X inhibits *Dux*-mediated ZGA gene expression through direct binding at the *Dux* locus. Additionally, two subfamilies of TEs, ERVK and ERVL, were misregulated. ERVL is directly activated by *Dux* and is therefore indirectly repressed by H2A.X. In contrast, ERVK may be regulated by indirect activation by H2A.X.

We also demonstrated that H2A.X is the major H2A variant deposited into chromatin in both mouse and human cleavage-stage embryos with ZGA activity. In mice, major ZGA occurs at the 2-cell stage, whereas in humans, major ZGA occurs in the 4-8 cell cleavage stage. Our current research demonstrated that the histone variant H2A.X showed an enrichment trend in human cleavage-stage embryos. Combined with previous studies that found that H2A.X is specifically expressed in mouse embryos at the 1-2 cell stage [25], all of these results indicate that H2A.X may play a special role in the process of maternal to zygotic transition (Table 1). We propose that proper amount of H2A.X ensures ZGA genes and TEs to be at relatively normal expression levels, and in preimplantation embryos, dynamic H2A.X incorporation at ZGA/TEs loci may finely modulate expression of ZGA/TEs for developmental progression. Previously, we found that histone H3 variant H3.3 played an inhibitory role in regulating ZGA genes [41]. Therefore, we propose that H2A.X/H3.3-containing nucleosomes may have unique chromatin structures that may feature totipotency and ZGA events. It has been reported that H3.3/H2A.Z-containing nucleosomes are relatively more unstable to allow accessibility of transcription factors [42], and unique features

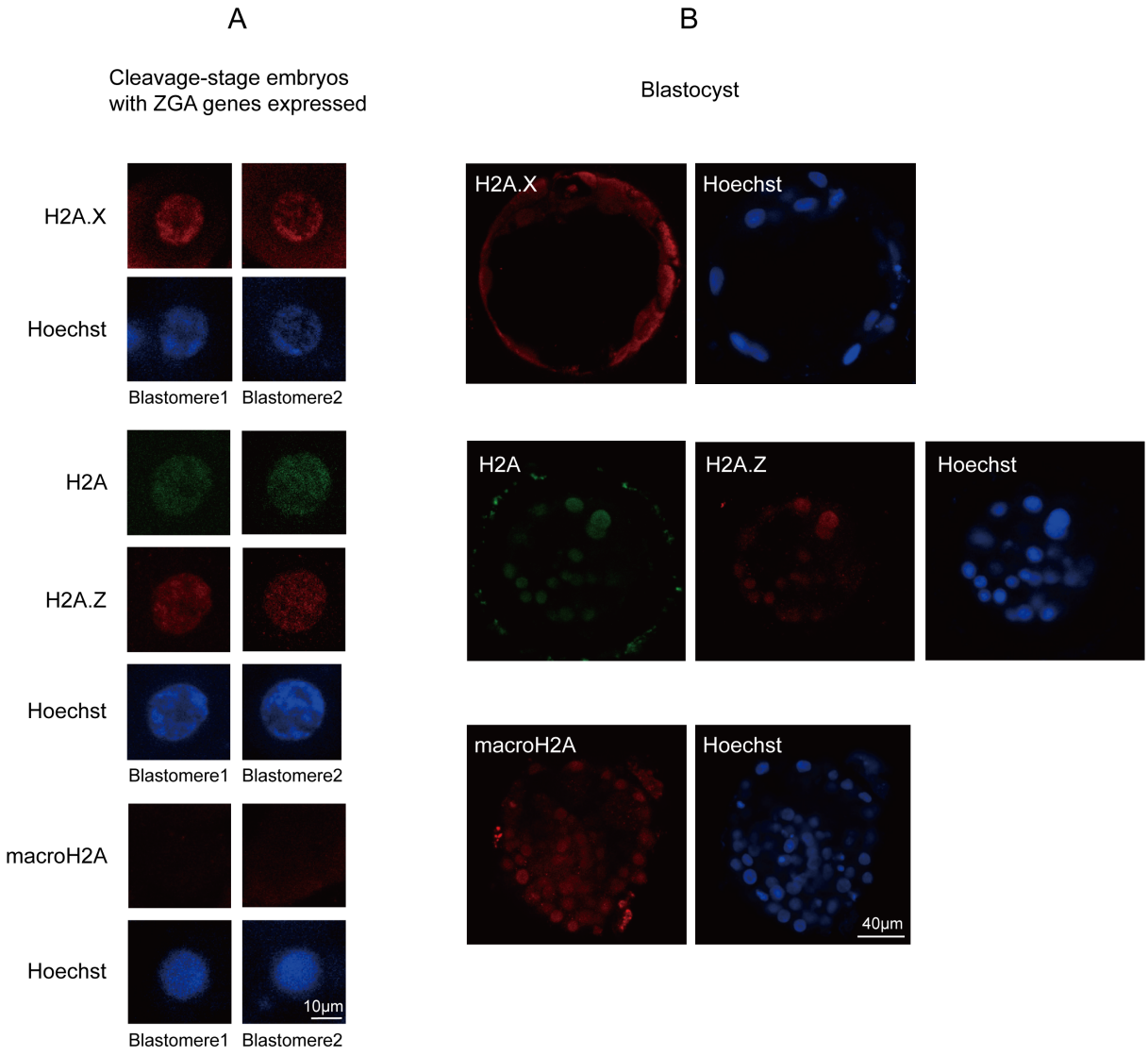

Fig. 3. Representative images of H2A variant deposition in early human embryos. Human cleavage-stage embryos (A) and blastocysts (B) were collected and stained with antibodies to different H2A variants, including H2A.X (n = 5 and 4, respectively), H2A and H2A.Z (co-stained; n = 6 and 5, respectively), and macroH2A (n = 5 and 4, respectively). Hoechst stain was used to stain the DNA.

Table 1. Comparison of expression pattern of H2A variants between human and mouse

IF signal of H2A variant	Cleavage stage (ZGA stage)		Blastocyst stage	
Human	H2A.X	Strong	H2A.X	Strong
	H2A	Strong	H2A	Strong
	H2A.Z	Strong	H2A.Z	Strong
	macroH2A	undetectable	macroH2A	Strong
Mouse [34]	H2A.X	Strong	H2A.X	Weak
	H2A	Weak	H2A	Strong
	H2A.Z	undetectable	H2A.Z	Strong
	macroH2A	undetectable	macroH2A	Strong

may also apply to H3.3/H2A.X-containing nucleosomes.

Future studies should include how H2A.X represses ZGA in mESCs, functions of phosphorylated H2A.X in regulation of ZGA and TEs through point mutagenesis in mESCs, characteristics and physical structures of H2A.X-containing nucleosomes in early mouse embryos, and elucidating chromatin associations of different H2A variants in mouse and human early embryos.

Collectively, our results show that H2A.X regulates cleavage embryo genes and TEs in mice, and may also be present in humans because of its abundance in human cleavage embryos (Supplementary Fig. 2).

Conflict of interests: The authors declare no conflict of interest.

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