Female human primordial germ cells display X-chromosome dosage compensation despite the absence of X-inactivation

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X-chromosome dosage compensation in female placental mammals is achieved by X-chromosome inactivation (XCI). Human pre-implantation embryos are an exception, in which dosage compensation occurs by X-chromosome dampening (XCD). Here, we examined whether XCD extends to human prenatal germ cells given their similarities to naive pluripotent cells. We found that female human primordial germ cells (hPGCs) display reduced X-linked gene expression before entering meiosis. Moreover, in hPGCs, both X chromosomes are active and express the long non-coding RNAs X active coating transcript (XACT) and X inactive specific transcript (XIST)—the master regulator of XCI—which are silenced after entry into meiosis. We find that XACT is a hPGC marker, describe XCD associated with XIST expression in hPGCs and suggest that XCD evolved in humans to regulate X-linked genes in pre-implantation embryos and PGCs. Furthermore, we found a unique mechanism of X-chromosome regulation in human primordial oocytes. Therefore, future studies of human germine development must consider the sexually dimorphic X-chromosome dosage compensation mechanisms in the prenatal germline.

Dosage compensation of genes on the X chromosome is an essential epigenetic event that equalizes the X-linked gene imbalance between males and females. In mice, dosage compensation is mediated by XCI, which is established early during development, first in an imprinted form and, after a brief phase of reactivation in naive pluripotent epiblast cells, through the random form by silencing either the maternal or paternal X chromosome. Molecularly, XCI is mediated by the long non-coding RNA (lncRNA) XIST, which coats the X chromosome in cis to establish and maintain a silencing compartment over the X-chromosome territory. Once established, XCI is stably maintained in female somatic cells; however, in female mouse primordial germ cells (mPGCs), the inactive X chromosome (Xi) is reactivated, which coincides with global epigenetic reprogramming. Following epigenetic reprogramming, and as mPGCs differentiate into meiotic cells in females or prospermatogonia in males, germ cells display X-chromosome dosage excess or X-chromosome dosage decompensation, respectively, highlighting the sexually dimorphic regulation of gene expression on the X chromosome in mouse germ cells.

Despite the conservation of XIST and XCI across placental mammals, it is now appreciated that there is no evidence for the imprinted form of XCI in human pre-implantation embryos. Moreover, in human female pre-implantation blastocysts, XIST is uniquely expressed from both X chromosomes and the expression of X-linked genes on both alleles is transcriptionally reduced but not silenced—a compensated state that is referred to as XCD.

Another striking difference in humans is the existence of XACT, a primate-specific lncRNA that is expressed from the active X chromosome(s) in both human pre-implantation embryos and pluripotent stem cells that can oppose the function of XIST. The expression status of XACT in hPGCs is unknown.

The unique XCD state in human pre-implantation embryos has been puzzling and is speculated to resolve to XCI within 1–2 weeks. It has therefore been postulated that the transient accumulation of XIST on both X chromosomes with XCD represents the initiating stages of the normal process of XCI. This interpretation is consistent with the biallelic XIST expression that is observed in differentiating female mouse embryonic stem cells (ESCs) during the XCI initiation process, yet is inconsistent with other findings that suggest that there is a transient state with two active X chromosomes (Xa) without XIST expression in cells transitioning between XCD and XCI. As mouse and human PGCs capture many of the epigenetic features of epiblast cells from the pre-implantation embryo, yet hPGCs stably persist for around two months during development, we hypothesized that female hPGCs could serve as an alternative model to evaluate the possibility that XCD is an independent and stable regulatory mechanism for X-chromosome dosage compensation in humans.

Results

Female hPGCs express XACT from two active X chromosomes. As the expression of the X-linked lncRNA XACT is associated...
with the unique X-chromosome state of human pre-implantation embryos\textsuperscript{1,4,5}, we performed RNA fluorescence in situ hybridization (FISH) analysis of \textit{XACT} in prenatal ovaries together with immunostaining for the germ cell-specific protein deleted in azoospermia like (DAZL), which marks hPGCs\textsuperscript{37}. We observed \textit{XACT} transcripts in nearly all female hPGCs (DAZL\textsuperscript{+} cells) at weeks 7–8 post-fertilization (p.f.), whereas somatic cells (DAZL\textsuperscript{−}) lacked \textit{XACT} expression (Fig. 1a,b). We identified two \textit{XACT} clouds in approximately 60–70% of hPGCs; each of these \textit{XACT} clouds was probably associated with the X chromosome that the RNA was transcribed from (Fig. 1c). Starting from 10 weeks p.f., hPGCs heterogeneously differentiate into meiotic germ cells in females\textsuperscript{24}, repressing pluripotency-related genes such as \textit{NANOG}\textsuperscript{25}. We therefore also detected \textit{NANOG} to distinguish between \textit{NANOG}\textsuperscript{+}DAZL\textsuperscript{−} hPGCs and \textit{NANOG}\textsuperscript{−}DAZL\textsuperscript{+} meiotic germ cells. We discovered that \textit{XACT} is still expressed in the majority of \textit{NANOG}\textsuperscript{+}DAZL\textsuperscript{−} female hPGCs at week 14 p.f. (Fig. 1d,e). By contrast, the majority of \textit{NANOG}\textsuperscript{−}DAZL\textsuperscript{+} meiotic germ cells are \textit{XACT} negative (Fig. 1d,e). Thus, \textit{XACT} is expressed from both X chromosomes in hPGCs, and is not expressed by ovarian somatic cells.

To evaluate whether additional genes encoded on the X chromosome are biallelically expressed in hPGCs, we performed RNA FISH analysis of the X-linked genes \textit{HUWE1} and \textit{Atrx}, detecting their nascent transcription foci in week 8 p.f. ovaries. We found that the majority of hPGCs marked by \textit{XACT} also biallelically expresses \textit{HUWE1} and \textit{Atrx} (Fig. 1f–h). By contrast, the somatic cells express these genes from a single allele due to XCI (Fig. 1f–h). These results are consistent with previous allelic analysis of X-linked gene expression from 53 sorted female germ cells\textsuperscript{30}, which revealed biallelic expression of X-linked genes (Extended Data Fig. 1a). As a consequence, we conclude that female hPGCs carry two active X chromosomes.

To confirm the active state of the X chromosome in female hPGCs, we assessed trimethylation of Lys 27 of histone H3 (H3K27me3), a chromatin mark that specifically accumulates on the Xi\textsuperscript{31,32}. In mice, we assessed H3K27me3 on the Xi at 2–3 p.f.\textsuperscript{34}, it is not possible to study hPGC specification in vivo. We therefore modelled hPGC specification using the differentiation of hPGC-like cells (hPGCLCs)\textsuperscript{35} from male (UCLA2 ESC)\textsuperscript{36} and female (MZT04-J iPSC) pluripotent stem cells (PSCs), by analysing \textit{XACT} expression in ITGA6\textsuperscript{+}EPCAM\textsuperscript{+} hPGCLCs and somatic cells at day 4 of differentiation (Fig. 2a). We discovered that most male hPGCLCs had one \textit{XACT} cloud, whereas most female hPGCLCs had two (Fig. 2b–g). By contrast, >90% of the somatic cells were \textit{XACT} negative (Fig. 2b–g). The detection of biallelic \textit{XACT} in female hPGCLCs is consistent with maintenance of the eroded Xi in differentiating hESCs\textsuperscript{31}. Collectively, these data uncover that the lncRNA \textit{XACT} is a new marker of male and female hPGCs in vivo and in vitro.

\textbf{\textit{XACT} expression is epiblast specific in human embryo attachment cultures.} Given that \textit{XACT} is expressed in \textit{NANOG}\textsuperscript{+} hPGCs and hPGCLCs, we next investigated \textit{XACT} distribution during the peri-implantation window of human development using human embryo attachment culture\textsuperscript{14,36}. Specifically, we explored the pattern of \textit{XACT} expression in \textit{NANOG}\textsuperscript{+} epiblast cells versus \textit{NANOG}\textsuperscript{−} trophectoderm (TE) and primitive endoderm (PE) cells. We also discovered the lncRNA \textit{XIST} to uncover changes in its distribution. First, we performed RNA FISH for \textit{XACT} and \textit{XIST} in combination with immunostaining for \textit{NANOG} in female and male human blastocysts (day 6 p.f.). At day 6 p.f., both \textit{XACT} and \textit{XIST} clouds could be identified on all X chromosomes (one in males and two in females) in 75% of female and 55% of male \textit{NANOG}\textsuperscript{+} epiblast cells (Fig. 3a,b,d,e,f). Furthermore, 30% of male epiblast cells also express only \textit{XACT} (Fig. 3e). By contrast, \textit{NANOG}\textsuperscript{−} TE and PE cells have more diverse \textit{XIST} and \textit{XACT} expression states—\textit{XACT} is more often repressed (Fig. 3c,f). These observations indicate that \textit{XACT} expression is more strongly associated with the \textit{NANOG}\textsuperscript{+} epiblast. Using human embryo attachment culture\textsuperscript{26,39} grown to day 12 p.f.—that is, the legal limit for human embryo culture in California—we discovered that the majority of female \textit{NANOG}\textsuperscript{+} epiblast cells continued to display \textit{XIST} and \textit{XACT} clouds on both X chromosomes (Fig. 3g,3h), whereas male epiblast cells maintained expression of \textit{XACT} but not \textit{XIST} (Fig. 3i,k). In the majority of female \textit{NANOG}\textsuperscript{−} cells, \textit{XACT} was repressed and \textit{XIST} was expressed from one X chromosome (Fig. 3i), suggesting that these cells have transitioned to the initiation of XCI. By contrast, in male embryos, both lncRNAs were silenced in 70% of \textit{NANOG}\textsuperscript{−} cells (Fig. 3i). Together, these data reveal that \textit{XACT} is expressed by the majority of \textit{NANOG}\textsuperscript{+} epiblast cells in the pre-implantation and early peri-implantation stages of male and female human embryo development. By contrast, \textit{XIST} is rapidly repressed in male \textit{NANOG}\textsuperscript{+} epiblast between days 6 and 12, highlighting differences in \textit{XIST} regulation in male and female human peri-implantation development.

\textbf{Female hPGCs dampen expression from the active X chromosomes.} As female hPGCs express \textit{XACT} from both X chromosomes, our next goal was to determine whether XCD occurs in hPGCs. We performed single-cell RNA sequencing (scRNA-seq) on single-cell suspensions of five prenatal ovaries and five prenatal testes from weeks 6 to 16 p.f. (Fig. 4a–c). This unbiased approach yielded a total of ∼50,000 prenatal gonadal cells, including ∼281 male and 1,938 female germ cells that were used to analyse the ratio of X-linked gene expression to autosome gene expression (X/A ratio; a summary of which is provided in Supplementary Table 1).

Calculating the X/A ratio across individual cells per developmental age revealed that female germ cells consistently had a higher X/A ratio compared with male germ cells (Extended Data Fig. 2a,b). This difference between female and male germ cells arises from the significantly lower X-linked gene expression in males (Extended Data Fig. 2c). By contrast, we found no difference in the X/A ratio between male and female gonadal somatic cells (Extended Data Fig. 2a,b). We confirmed these results using a published scRNA-seq dataset of c-KIT and size-selected germ cells\textsuperscript{40}, in which germ line cells are called fetal germ cells (FGCs) (Extended Data Fig. 2d,e).
Fig. 1 | Male and female hPGCs express the lncRNA XACT and female hPGCs carry two active X chromosomes in vivo.

a, Immuno-RNA FISH analysis of DAZL (green) and XACT (red) in a week 7 p.f. prenatal ovary with DAPI staining (blue) to detect nuclei. Scale bar, 30 μm. Inset zoom 1.2×. n = 1 pair of ovaries.

b, Quantification of cells with XACT clouds on the basis of the experiment shown in a. n = 100 cells.

c, Quantification of the number of XACT clouds in DAZL+ hPGCs at weeks 7 and 8 p.f. n = 100 cells per time point.

d, Immuno-RNA FISH analysis of NANOG (magenta), DAZL (green), XACT (red) and DAPI (grey) in a week 14 p.f. fetal ovary. n = 1 pair of ovaries. Insets: a NANOG−DAZL+ hPGC negative for XACT (top); and a NANOG+DAZL+hPGC with two XACT clouds (bottom). Scale bar, 30 μm. Inset zoom 2.5×. e, Quantification of the proportion of cells with different XACT cloud patterns in the hPGCs (NANOG+DAZL+) and differentiating hPGCs (NANOG−DAZL+) from d. n = 92 and n = 95 cells, respectively, were assessed.

f, g, Representative RNA FISH images for detecting nascent transcripts of the X-linked genes HUWE1 (f) and ATRX (g), which are both normally subject to XCI, in a week 8 p.f. ovary. hPGCs are marked by XACT expression. The experiments were performed twice with similar results. Scale bar, 15 μm.

h, Signal quantification for g. n = 60 and n = 70 cells for ATRX and HUWE1, respectively.

i, Published bulk RNA-seq reads mapped to the XACT genomic locus in female (F) hPGCs (red, isolated using c-KITbright or using INTa6/EpCAM), male (M) hPGCs (blue, enriched for TNAP/KIT expression) and gonadal somatic cells (grey). Chr., chromosome.

j, Immuno-RNA FISH of XACT (red), NANOG (green) and DAPI (blue) in fetal male testes at week 13 p.f. n = 1 pair of testes. Scale bar, 30 μm. Inset zoom 2.5×.

k, Quantification of the proportion of cells with one XACT cloud in NANOG+ male hPGCs from j. n = 75 cells. Source data are available online.
Fig. 2 | The IncRNA XACT is restricted to male and female hPGCLCs and is not expressed in somatic cells in vitro. a, Differentiation of hPGCLCs from human induced PSCs (hiPSCs) or hESCs through an incipient mesoderm-like cell (iMeLC) intermediate. PGCLCs and somatic cells within the aggregates were separated at day 4 by fluorescence-activated cell sorting (FACS) using antibodies against EPCAM and ITGA6. b, RNA FISH analysis of XACT in primed female hiPSCs (MZT04-J) that harbour an Xa and an eroded X chromosome with XACT (red) and DAPI (blue) staining. XACT clouds were detected from the Xa and eroded X chromosome. Scale bar, 20 μm. Inset zoom 2.5×. The experiments were performed twice with similar results. c, Female hiPSCs were differentiated to hPGCLCs and isolated from the aggregates using FACS at day 4 (left). The hPGCLC population is indicated. Right, XACT RNA FISH analysis of hPGCLCs and somatic cells. The experiments were performed twice with similar results. Scale bar, 10 μm. d, Quantification of the proportion of cells with different numbers of XACT clouds in starting hiPSCs (n=100 cells), hPGCLCs (n=82 cells) and somatic cells (n=100 cells) from c. e, RNA FISH analysis of XACT (red) in male hESCs (UCLA2). Scale bar, 20 μm, inset zoom 2.5×, similar to b. f, FACS (left) and XACT RNA FISH (right) analyses as described in c, except for with UCLA2 hESCs; the experiments were performed twice with similar results. Scale bar, 10 μm. g, Quantification of the proportion of cells with a different number of XACT clouds in UCLA2 hESCs (n=100 cells) and derived hPGCLCs (n=92 cells) and somatic cells (n=100 cells pooled from the two experiments). Source data are available online.
Thus, the X/A ratio is higher in female germ cells relative to male germ cells, whereas male and female gonadal somatic cells are equivalent.

As germ cell differentiation into meiotic cells is heterogeneous, we created an unsupervised developmental trajectory, which ordered female human germ cells across 11 clusters (Fig. 4d,e). Clusters 0–5 represent hPGCs expressing the transcription factors NANOG and OCT4, together with the hPGC markers NANOS3, PRDM1 and SOX17 (Fig. 4d). Starting in cluster 6, we observed downregulation of naive-like pluripotency genes and upregulation of the meiotic licensing gene STRA8 (ref. 44) and of genes encoding RNA-binding proteins such as DAZL and DDX4 (also known as VASA; clusters 6–7). This was followed by expression of the meiotic prophase I genes SPO11 and SYCP1 in clusters 8–9 and, ultimately, in cluster 10, upregulation of primordial oocyte genes including the zona pellucida protein 3 (ZP3) (Fig. 4d). Thus, consistent with previous reports, our data capture the heterogenous differentiation of female hPGCs into meiotic germ cells beginning around week 9–10 p.f., which results in a complex mixture of germ cells including hPGCs, meiotic germ cells and primordial oocytes in a given prenatal ovary (Fig. 4f).

Analysis of the X/A ratio in female germ cells along the developmental trajectory (Fig. 5a) showed that, as hPGCs begin differentiating into meiotic germ cells (cluster 6 onwards), the X/A ratio increases, reaching maximal levels in cluster 9 before precipitously dropping in primordial oocytes (cluster 10). Moreover, the X/A ratio in hPGCs (clusters 0–5) is lower than in gonadal somatic cells (Fig. 5a and Extended Data Fig. 3a–c). The median X/A ratio in hPGCs (clusters 0–5) is significantly lower compared with meiotic germ cells (clusters 6–9) but higher compared with primordial oocytes (cluster 10; Fig. 5b). These changes were largely due to changes in X-linked gene expression (Extended Data Fig. 3d,e). We confirmed these observations in the FGC scRNA-seq data. Similar to our analysis, female FGCs displayed a slightly lower X/A ratio compared with the X/A ratio in gonadal somatic cells in the hPGC state, as well as a subtle, albeit significant, increase in the X/A ratio after expression of STRA8 (Extended Data Fig. 3f,g). On the basis of these results, we conclude that the dosage of X-linked genes is dampened in female hPGCs and that this dosage compensation is erased as cells enter prophase I of meiosis I.

To evaluate X/A ratios in male prenatal germ cells, we ordered the cells along a developmental trajectory, which divided the male germ cells from week 6–16 p.f. gonads into five clusters (0–4)—the hPGC program corresponded to clusters 0–3 and cluster 4 captured differentiating germ cells (prospermatogonia; Extended Data Fig. 4a,b). In contrast to female germ cells that exhibit X-chromosome dosage excess with exit from the hPGC state, the X/A ratio does not change when male hPGCs differentiate into prospermatogonia (Extended Data Fig. 4c–h), which was confirmed with published datasets (Extended Data Fig. 4i,j). Thus, an increase in X/A ratio as hPGCs differentiate is a female-specific phenomenon that is associated with entrance into prophase I of meiosis I.

**XCD in female hPGCs is associated with XIST expression.** To evaluate whether XCD in female hPGCs is associated with the expression of XIST, as has been shown in female human pre-implantation embryos, we examined XIST in individual female germ cells along the developmental trajectory. We discovered that XIST expression is significantly higher in hPGCs (clusters 0–5) compared with meiotic germ cells (clusters 6–9) and primordial oocytes (cluster 10; Fig. 5c,d). This result was also validated in the female FGC dataset (Extended Data Fig. 5a). In agreement with the low expression of XIST in primordial oocytes, the levels of the RNA are also low in adult oocytes (Extended Data Fig. 5b). Thus, the increase in the X/A ratio from hPGCs to meiotic germ cells is accompanied by a decrease in XIST transcript levels (Fig. 5b,d and Extended Data Fig. 5c). Consistent with this result, when analysing hPGCs and meiotic germ cells together (clusters 0–9) on the basis of XIST expression, XIST+ cells displayed a significantly lower X/A ratio compared with XIST− cells. Primordial oocytes (cluster 10) have an even lower X/A ratio, yet express XIST at the lowest level (Fig. 6b,d and Extended Data Fig. 5d).

Next, we evaluated the localization of XIST in female hPGCs using RNA FISH from week 7 to week 14 p.f., using XACT as a marker of hPGCs. We found that XIST is detectable in the vast majority of XACT-expressing hPGCs, with diverse patterns of the XIST signal (Fig. 6a–c). These include (1) an eroded cloud pattern, whereby XIST is restricted over one X chromosome in a pattern that is characteristic of Xi localization, yet less enriched compared with the Xi in somatic cells, combined with a nascent transcription spot of XIST on the second X chromosome; (2) a dispersed configuration whereby the XIST signal is detected throughout a large portion of the nucleus albeit in the vicinity of both XACT clouds, suggesting that XIST is expressed from both X chromosomes; (3) one dot in the vicinity of one of the two XACT signals, indicating expression from one X chromosome; and (4) two dots representing the nascent transcription sites of XIST on both X chromosomes (Fig. 6a). Quantification of the XIST expression patterns in female hPGCs with two XACT clouds—which enabled the localization of both X chromosomes—revealed that the majority of hPGCs (58%) at week 7 p.f. had a dispersed XIST signal (Fig. 6c). Around 6% of cells with biallelic XACT expression displayed an eroded XIST cloud pattern and 19% and 15%, respectively, exhibited mono- and biallelic nascent XIST transcription foci (Fig. 6c). At later stages of embryo development (week 8 and week 14 p.f.), XACT+ female hPGCs displayed similar patterns of XIST RNA with an increasing fraction of XIST+ cells (Fig. 6b,c).

As we observed an X/I-like distribution of XIST in 6% of week 7 and 8 p.f. hPGCs with two XACT clouds, we tested whether female

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**Fig. 3 | XACT is predominantly expressed in NANOG+ pre- and post-implantation epiblast cells.** a,d,g,i. Immuno-RNA FISH analysis of NANOG (white), XIST (green) and XACT (red) in day 6 p.f. intact female pre-implantation blastocysts (a) (n = 2 blastocysts); day 6 p.f. male pre-implantation blastocysts (d) (n = 3 blastocysts); female embryos cultured to day 12 p.f. using human embryo attachment culture (g) (n = 3 embryos); and male human embryos cultured to day 12 p.f. using human embryo attachment culture (i) (n = 2 blastocysts). Scale bars, 30 μm. For a, d, g and i, insets: Nanog+ (blue) nuclei with XIST and XACT clouds. Inset zoom 7.5 x. b. Quantification of the proportion of cells with different numbers of XIST and XACT clouds in NANOG+ epiblast cells from the female blastocysts shown in a. c. 17 cells were analysed in 2 blastocysts. c. Quantification of the RNA pattern in NANOG+ TE and PE cells from the female blastocyst shown in a. n = 111 cells quantified from the 2 blastocysts. d. Quantification of the proportion of cells with different numbers of XIST and XACT clouds as described in b from the NANOG+ epiblast cells from the male blastocysts shown in d. n = 24 cells from 3 blastocysts counted. f. Quantification of the RNA pattern as described in c, except for with NANOG- TE and PE cells from the male blastocysts shown in d. n = 180 cells from 3 blastocysts were counted. h. Quantification of the proportion of cells with different numbers of XIST and XACT clouds as described in b for the female day 12 embryos from g. n = 67 cells from 3 embryos were counted. j. Quantification of the RNA pattern as described in c for the female day 12 embryos shown in g. n = 180 cells from 3 embryos counted. f. Quantification of the proportion of cells with different numbers of XIST and XACT clouds as described in b for the male day 12 embryos from j. n = 70 cells from 2 embryos were assessed. l. Quantification of the RNA pattern as described in c for the male day 12 embryos in j. n = 120 cells from 2 embryos were quantified. Source data are available online.
hPGCs with one XACT cloud represent cells that have undergone XCI. In this case, XACT should be expressed from the Xa and XIST from the Xi. However, we found that XIST is typically expressed from the same X chromosome as XACT in cells with monoallelic XIST/XACT expression (Fig. 6d), providing additional evidence for XCD instead of XCI in female hPGCs.
Fig. 4 | Female germ cells undergo dynamic and ordered transcriptional changes between 7-16 weeks p.f. a, The distribution of single-cell data derived from scRNA-seq from five prenatal male and five prenatal female gonads from 6–16 weeks p.f., displayed on a UMAP plot. n = 49,674 cells. b, Annotation of the gonadal cell types in the map in a on the basis of the expression of cell-type-specific markers. c, The distribution of male and female cells on the map from a, d, Ordering of female germ cells along the developmental trajectory from cluster 0 to cluster 10, with classification into hPGCs (clusters 0–5), meiotic germ cells (clusters 6–9) and primordial oocytes (PO, cluster 10) on the basis of diagnostic germ cell marker gene expression. Each cluster contains many individual cells (columns), for which expression of the indicated marker is given (rows). n = 1,938 cells from n = 5 samples. e, Female germ cells displayed on a UMAP plot, labelled by their cluster assignment from d. f, For each of the five female gonads described in a, the proportion of cells in the clusters defined in d is given. These data show that the repression of the pluripotency expression program and meiotic licensing (expression of STRA8) begins between weeks 9 and 10 p.f., and that all germ cells at week 7 are in the hPGC state. Source data are available online.
To confirm that the loss of XIST expression was associated with the differentiation of NANOG+DAZL+ hPGCs into NANOG−DAZL+ meiotic germ cells, we evaluated a fetal ovary at 14 weeks p.f. using RNA FISH. We found that XIST was detectable in the majority of NANOG+DAZL+ hPGCs, with the dispersed pattern being most prominent. By contrast, the majority of NANOG−DAZL+ germ cells were negative for XIST and the subset of cells with XIST expression displayed the dot-like distribution (Fig. 6e,f). The quantification suggests that cells transition from the dispersed XIST pattern to the two-XIST-dot and one-XIST-dot patterns before XIST is turned off during germ cell differentiation. Furthermore, evaluating XIST localization around the X chromosomes in female hPGCs relative to female human pre-implantation embryos revealed a higher degree of XIST dispersal in hPGCs (compare Figs. 3 and 6), suggesting that the association between XIST and chromatin differs between the two cell types.

In addition to detecting XIST expression in female hPGCs, XIST transcripts could also be detected in male hPGCs on the basis of scRNA-seq data, albeit at much lower level than in female cells (Extended Data Fig. 5e,f). Furthermore, a small but significant reduction in the X/A ratio in male hPGCs was correlated with a significant increase in XIST levels (Extended Data Figs. 4j and 5f). Thus, a subset of male hPGCs can express XIST and display slight dampening of X-chromosome dosage. Moreover, female germ cells that lacked XIST displayed an increase, albeit not significant, in the XACT cloud size compared with cells with XIST expression (Extended Data Fig. 5g). Taken together, these data suggest that XIST may mediate dampening of X-linked gene expression in hPGCs.

**Discussion**

Here, by analysing human pre-implantation embryos, human embryo attachment culture, hPGCLC differentiation in vitro and...
hPGCs in vivo, we revealed that the lncRNA XACT is expressed in pluripotent epiblast cells and hPGCs/hPGCLCs (Fig. 6g and Extended Data Fig. 6). Mechanistically, this result may be explained by the presence of an enhancer that threads XACT into the pluripotency network common to these cell types45. From this analysis, we describe that XACT is a unique marker of hPGCs, and speculate that it could be used to trace hPGCs from the time of lineage specification using RNA FISH. Moreover, our RNA FISH analysis of the X-linked genes XACT, ATRX and HUWE1, together with absence of H3K27me3 accumulation in the nucleus of most female hPGCs demonstrates that female hPGCs harbour two Xa chromosomes from at least week 4 p.f. onwards.
Fig. 6 | XIST is repressed as hPGCs enter meiosis. a, RNA FISH analysis of XIST (green) and XACT (red) with DAPI (blue) identifying nuclei. n = 1 pair of ovaries (week 7 p.f.). Scale bar, 30 μm. Inset zoom 2.1x. b, RNA FISH as described in a, except for week 14 p.f. ovaries. n = 1 pair of ovaries, scale bar, 30 μm. Inset zoom 2.6x. c, The proportion of cells with the indicated expression patterns in female hPGCs with two XACT clouds. For each time point, n = 100 cells from 1 pair of ovaries. d, The proportion of cells with the indicated XIST expression patterns, similar to as described in c except in female hPGCs with a single XACT cloud. Cells with a dispersed XIST signal for which it was not clear whether XIST was expressed from one or both alleles were included in the green category. Total of n = 42 cells from 3 ovaries. e, Immuno-RNA FISH analysis of NANOG, DAZL and XIST RNA in week 14 p.f. ovaries (1 pair of ovaries). Scale bar, 30 μm. f, The proportion of cells with the indicated XIST expression patterns similar to in e in female hPGCs (NANOG*-DAZL*) and meiotic germ cells (NANOG*-DAZL*) from n = 104 and n = 98 cells, respectively from 1 sample. g, Female NANOG* pre-implantation epiblast (pre-Epi) and post-implantation epiblast (post-Epi) cells predominantly express XACT and XIST from both X chromosomes. Although genes on both X chromosomes in pre-implantation epiblast cells are dampened due to XCD (Xδ)15, it is unclear whether XCD also occurs in female post-implantation epiblast cells. However, expression of both XACT and XIST from both X chromosomes indicates that XCI has not yet occurred. NANOG* female hPGCs, similar to post-implantation epiblast cells, exhibit XCD with expression of XIST and XACT from both alleles. Notably, XIST is more dispersed in the nucleus of hPGCs (shown by the glow around the X chromosome) compared with pre/post-implantation epiblast cells. After advancement to meiosis, female germ cells silence both XACT and XIST and upregulate X-linked gene expression, transitioning to the Xa state. Next, in primordial oocytes, the X/A ratio is reduced to lower than in female hPGCs without expression of XIST. We call this XIST-independent repression of the X/A ratio oocyte-specific X-chromosome regulation (XO). Male hPGCs harbour an Xa but exhibit a lower X/A ratio compared with male somatic cells, and this state is retained after differentiation into spermatogonia. We refer to this state as Xα*. Gonadal somatic cells display XCI at each stage analysed.

Although our imaging approaches demonstrated the presence of two active X chromosomes in female hPGCs, the scRNA-seq data revealed that the X/A ratio is reduced in female hPGCs compared with female meiotic germ cells (Fig. 6g). These results indicate that X-linked dosage compensation in female hPGCs is regulated by the XCD mechanism, similar to female naive human pluripotent stem cells12 and female human pre-implantation embryos4. Although XCD is a transient state in pre-implantation embryos, in the case of hPGCs, we show that XCD is not a transitional state into XCI, but rather a stable state that lasts for at least 6 weeks until the point of meiotic initiation (Fig. 6g). Similar to female mouse PGCs, we also show that the X/A ratio excess occurs as female hPGCs initiate meiosis. After prophase I of meiosis I, the X/A ratio quickly declines coincident with primordial oocyte formation indicating a third unique state of X-chromosome dosage compensation, which we have called oocyte-specific X-chromosome regulation (XO; Fig. 6g). Interestingly, the loss of XCD in the female germline after meiotic entry is linked to the silencing of XIST (Fig. 6g), suggesting that XIST is the mediator of XCD. By contrast, the further decline in the X/A ratio in primordial oocytes occurs in the absence of XIST expression. It remains unclear whether this regulation is achieved by XCD or other mechanisms.

Given that male hPGCs have a reduced X/A ratio compared with female and male somatic cells and female hPGCs, we refer to the active X chromosome in male prenatal germ cells as Xα* (Fig. 6g). The lower X/A ratio in male hPGCs relative to female hPGCs could be due to inefficient dampening of X-linked gene expression from both X chromosomes by XIST in female hPGCs, such that the levels in female hPGCs are higher than males. In support of this hypothesis, XIST is highly dispersed in female hPGCs, which may lead to less efficient XCD compared with the more cloud-like distribution of XIST reported for the pre-implantation embryo15,16. By contrast, a higher X/A ratio in female and male somatic cells could be explained by upregulation of single Xα in somatic cells12,46,47, which may not occur in female or male hPGCs. Combined with XCD occurring on the X chromosomes in female hPGCs, this alternative explanation would explain the lower X-linked gene expression in female hPGCs compared with somatic cells.

As germline specification in humans takes days compared with hours in mice, and hPGC development is a much longer process than in mice, it is conceivable that the maintenance of X-chromosome dosage compensation in germ cells between the two species is different. It is probable that a primate-specific X-chromosome regulation mechanism might be necessary to compensate dosage of X-linked genes in the human embryo during PGC specification and the first trimester of pregnancy. However, how XIST and XACT contribute to X-chromosome gene regulation in the developing human germline will need to be studied mechanismically. Achieving this goal will require new in vitro cell models of hPGC development that have the ability to reliably promote the differentiation of hPGCLCs into meiotic germ cells and primordial oocytes combined with functional approaches17.

X-chromosome dosage regulation might be extremely important for patients with Turner (XO) and Klinefelter (XXY) syndrome, who have infertility associated with loss of germine cells18,19. Although germline development in fetuses diagnosed with Turner syndrome is morphologically normal, oocyte loss occurs within the first few months after birth1. Potentially, meiosis does not occur correctly in patients with Turner syndrome due to diminished levels of critical X-linked genes in differentiating XO hPGCs compared with XX hPGCs. Thus, upregulation of X-linked gene expression with entrance into meiosis may be necessary for the formation of mature oocytes.

In summary, with the demonstration of XCD, our research draws parallels between the X-chromosome state of human epiblast and hPGCs. Our study sheds light on mechanisms that regulate X-linked gene expression in hPGCs before meiosis, and reveals a unique X-chromosome state in oocytes, which could potentially be important for oocyte formation and zygote development downstream.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41556-020-00607-4.

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Methods

Human fetal tissues. Prenatal gonads (4–16 weeks p.f.) were obtained from either the University of Washington Birth Defects Research Laboratory (BDRL) or the University of Tübingen. At the BDRL, prenatal gonads were obtained with regulatory oversight from the University of Washington IRB approved Human Participants protocol, combined with a Certificate of Confidentiality from the Federal Government. BDRL collected the fetal testes and ovaries and shipped them overnight in HBSS with an ice pack for immediate processing at UCLA. Prenatal samples from the University of Tübingen were delivered to UCLA 24–48h after the procedure. The research project was also approved by the research ethics committee of the University of Tübingen (IRR, 584/2018BO2 and 634/2017BO1). All human fetal tissue used here was obtained following informed consent. The donated human fetal tissue sent to UCLA did not carry any personal identifiers. No payments were made to donors and the donors knowingly and willingly consented to provide research materials without restrictions for research and for use without identification. Developmental stages were documented by BDRL and the University of Tübingen as days p.f. using a combination of prenatal intakes and Carnegie staging. A total of 16 fetal samples was used for this study.

Human pre-implantation embryos. The use of human embryos in this research project followed California State law and was reviewed by the Institutional Review Board (IRB) and the human embryonic stem cell research committee (ESRCO) at UCLA. The ESRCO committee at UCLA approves human pluripotent stem cell and human embryo work at UCLA according to 2016 ISSCR guidelines. Together, these committees approve the process of informed consent and experimental design of human embryos for research purposes on an annual basis. Patients were not paid for participating, and all of the donors were informed that the embryos would be destroyed as part of the research study. All research in this study using human embryos complied with the principles that are laid out in the International Society for Stem Cell Research. Frozen human blastocysts at days 6–7 p.f. were used in this study and thawed using the VitKit Thaw (Irvine Scientific) according to manufacturer’s protocol. After thawing, embryos were cultured overnight in 5% O2, 6% CO2 at 37 °C. Embryos were washed with 1× PBS. Tissues were dissociated for 15 min at 37 °C. After every dissociation buffer containing of collagenase IV 10 mg ml−1 (Life Technologies, 17105041), DNase I 1:1,000 (Sigma-Aldrich, 4716728001), 10% fetal bovine serum (Life Technologies, 10098036) and 0.05% trypsin–EDTA (GIBCO, 25300-054) was included in the medium. The tissue suspension was filtered through a 40 μm strainer and counted using an automated cell counter (Becton Dickinson, FACSDiva™ Software BD FACSDiva™ Software BD FACSDiva™ Software BD FACSDiva™ Software). The cell concentration was adjusted to 800–1,200 cells per μl and immediately used for scRNA-seq.

Tissue processing for scRNA-seq. Fetal tissues were processed 24–48 h after termination. On arrival, tissues were gently washed with PBS and placed in dissociation buffer containing of collagenase IV 10 mg ml−1 (Life Technologies, 17104-019), dispase II 250 U ml−1 (Life Technologies, 17105041), DNase I 1:1,000 (Sigma-Aldrich, 4716728001), and 10% fetal bovine serum (Life Technologies, 10098036) × PBS. Tissues were dissociated overnight in 5% O2, 6% CO2, 37 °C, and the zona pellucida was removed with Tyrod's acidified solution (Irvine Scientific). A total of 28 human blastocysts were used here. The sex of the blastocysts was determined by cloud counts of IncRNA XIST and XACT expression from a single (male) or both X chromosomes (female).

Tissue processing for scRNA-seq. Fetal tissues were processed 24–48 h after termination. On arrival, tissues were gently washed with PBS and placed in dissociation buffer containing of collagenase IV 10 mg ml−1 (Life Technologies, 17104-019), dispase II 250 U ml−1 (Life Technologies, 17105041), DNase I 1:1,000 (Sigma-Aldrich, 4716728001), and 10% fetal bovine serum (Life Technologies, 10099141) in 1× PBS. Tissues were dissociated for 15 min at 37 °C. After every 5 min, the tissues were pipetted against the bottom of Eppendorf tube. Cells were subsequently centrifuged for 5 min at 500g, resuspended in 1× PBS with 0.04% BSA, strained through a 40 μm strainer and counted using an automated cell counter (Becton Dickinson, FACSDiva™ Software BD FACSDiva™ Software BD FACSDiva™ Software). The cell concentration was adjusted to 800–1,200 cells per μl and immediately used for scRNA-seq.

scRNA-seq library preparation and sequencing. scRNA-seq libraries were generated using the 10x Genomics Chromium systems, 314-BP, 50 ng ml−1 activin A (Peprotech, AF-120-14E), 3 mM CHIR99021 (Stemgent, 04-0004), 10 mM of ROCKi (Y27632, Stemgent, 04-0012-10) and 50 ng ml−1 primocin in Glasgow’s MEM (GMEM) (GIBCO, 11710-035). After 24 h, iMECs were dissociated into single cells with 0.05% trypsin–EDTA and plated into ultra-low cell attachment U-bottom 96-well plates (Corning, 3912) at a density of 5,000 cells per well in 200 μl of hPGCLC medium, which is composed of 15% KSR (GIBCO, 10828-028), 1x NEAA (GIBCO, 1110-050), 0.1 mM 2-mercaptoethanol (GIBCO, 21985-023), 1x penicillin– streptomycin–glutamine (GIBCO, 10378-016), 1 mM sodium pyruvate (GIBCO, 11360-070), 50 mM 2-mercaptoethanol (GIBCO, 25300-054), 1× PBS with 0.04% BSA, strained through a 40 μm strainer and counted using an automated cell counter (Becton Dickinson, FACSDiva™ Software BD FACSDiva™ Software). The cell concentration was adjusted to 800–1,200 cells per μl and immediately used for scRNA-seq.

scRNA-seq library analysis. scRNA-seq reads were aligned to the human hg38 genome assembly using 10x Genomics Cell Ranger v2.2. Expression matrices generated by Cell Ranger were imported into Scrapy for downstream analysis. First, all of the libraries were merged, and cells were filtered in the same manner. All of the genes that were expressed in less than five cells were discarded and cells with less than 250 expressed genes were excluded. The unique molecular identifier (UMI) counts were then normalized for each cell by the total expression, multiplied by 10,000 and log-transformed. Using Scrapy’s default method, highly variable genes were identified, and data were scaled to regress out variation from UMI counts and mitochondrial genes. Cells were clustered using the Louvain algorithm and the UMAP package was used to visualize cells in a two-dimensional plot. Germ cell clusters were identified by expression of germ-cell-specific markers, such as NANS5, DAZL, DDX4 and SYCP1. Gonadal somatic cells were annotated by previously published literature. The female and male germ-line trajectories were created by partition-based graph abstraction. The dataset of LI et al. was analysed through the same pipeline as described above. Gene expression matrices of female and male germ cells were exported from Scrapy and X/A ratio per cell was calculated using a custom R script.

Immunofluorescence. Slides of paraffin-embedded sections were deparaffinized by successive treatment with xylene and 100%, 70% and 50% ethanol. Antigen retrieval was performed by incubation with 10 mM Tris pH 9.0, 1× EDTA, 0.05% Tween-20 at 95 °C for 40 min. The slides were cooled and washed with 1× PBS and 1× TBS (PBS + 0.2% Tween-20). Cryosections and cryostat sections were fixed to lidded chambers fixed with 4% PFA for 30 min and then washed with 1× PBS. Paraffin-embedded sections, cryosections and cryostat sections were treated similarly. The slides were permeabilized with 0.5% Triton X-100 in 1× PBS, then washed with 1× TBS and blocked with 1% BSA in 1× TBS. Primary antibody incubation was conducted with 1% BSA for 1 h at room temperature. Samples were again washed with 3× TBS–Tween 20 and incubated with fluorescent secondary antibodies at 1:200 for 45 min, then washed and counterstained with DAPI for 5 min and mounted using Vectashield. A list of the primary antibodies used for immunofluorescence in this study is provided in Supplementary Table 1 under the antibody list tab. The secondary antibodies used in this study were all obtained from Life technologies and were used at 1:400 dilution. Images were taken using a LSM 880 Confocal Instrument (Zeiss) or a Nikon Ti-E. For image processing and analysis, Fiji (ImageJ) was used. For signal quantification, images were converted into 8-bit images and then analysed using profile plot tool. Intensity values were exported as a CSV file and then R Studio and the ggplot2 package was used for plotting.
RNA FISH. After sorting, hPGCLCs and non-hPGCLCs were attached to fibronectin-coated 18 mm circular glass coverslips (Thermo Fisher Scientific, 12-545-100) overnight. The next morning, the coverslips were washed with DPBS, fixed with 4% formaldehyde for 10 min, permeabilized with cold (4 °C) 0.5% Triton X-100 in DPBS for 10 min and serially dehydrated with cold (4 °C) 70–100% ethanol. Coverslips were air dried and hybridized with labelled DNA probes in a humidified chamber at 37 °C overnight, washed for three 5 min intervals with 50% formamide in 2x SSC, 2x SSC, then 1x SSC at 42 °C, counterstained with DAPI and mounted with Vectashield (Vector Labs, H-1000). Double-stranded DNA probes were generated from full-length cDNA constructs or bacterial artificial chromosome (BACs) as described previously 45. The following BACs were used: XIST (RP11-13M9), XACT (RP11-35D3), ATRX (RP11-1145J4) and HUWE1 (RP11-97SN19). Every new batch of probes was tested on normal human dermal fibroblasts before it was used in experiments.

Immuno-RNA FISH. Immuno-RNA FISH on cryosections was performed as described previously 39. In brief, immunostaining was performed first on cryosections and blastocysts as described above. Samples were post-fixed with 4% PFA after immunofluorescence staining, and RNA FISH was performed after post-fixation as described in RNA FISH section above.

Immuno-RNA FISH analysis of human blastocysts was performed as described previously 39 with the following modifications: embryos at day 5 and day 6 p.f. were thawed for these experiments and cultured for 24 h before staining. First, zona pellucida was removed with Tyrode’s acid and blastocysts were washed with Vogt’s BSA (Sigma-Aldrich) in RNAse-DNase-free DPBS. Blastocysts were then individually transferred to Ibbid chambers (Iibidi, 80827), which were coated with polylysine (Sigma-Aldrich, P4832-50ML). Fluid was aspirated until dry and the blastocysts were fixed with ice-cold 4% paraformaldehyde (PFA) for 10 min at room temperature. Immunostaining was performed as described above. In all buffers and antibody solutions, RNaseOUT 1:200 (Thermo Fisher Scientific, 10777019) was added to preserve RNA. Before performing RNA FISH, samples were post-fixed with 4% PFA for 10 min at room temperature. RNA FISH was then performed using DNA probes as described above.

Attached blastocyst culture. Human embryo attachment culture was performed according to previously described procedures 39,45. In brief, cryopreserved human blastocysts were received vitrified from the IVF clinic following consent and day 6 p.f. were thawed for these experiments and cultured for 24 h before thawing. Day 5 blastocysts were received vitrified from the IVF clinic following consent and cultured for 24 h before thawing. First, zona pellucida was removed with Tyrode’s solution acidified (Irvine Scientific) and plated onto an m-Slide eight-well chamber slide (Ibidi) in IVC-1 medium (Cell Guidance Systems) and incubated for 2 d at 37 °C and 5% CO₂ to allow for attachment. Medium was half replaced on the second and third day with IVC-1. From the fourth day onward, the medium was completely replaced with IVC-2 medium (Cell Guidance Systems) until the appropriate developmental day was reached up to a maximum of day 12, which includes the blastocyst stage plus days in culture. For these experiments, 9 blastocysts were cultured up to day 12 p.f., of which 5 were used for immuno-RNA FISH experiments.

Bulk RNA-seq data analysis. Published raw population RNA-seq datasets 30,46,53 of male and female hPGCs and somatic cells were downloaded and realigned to the hg19 genome as described previously 17 for lncRNA expression analysis. Expression tracks were generated using pyGenomeTracks package 57. Statistics and reproducibility. In the quantitative data, significance was assessed using Wilcoxon tests; \( P < 0.05, \text{**} P < 0.01, \text{***} P < 0.001; \text{ns}, \text{non-significant}. \) Statistical analyses are described in detail in the figure legends for each panel. Immuno-RNA FISH experiments were performed in two independent experiments with similar results, unless specified otherwise in the legends. scRNA-seq datasets were pooled from ten independent experiments. No statistical methods were used to determine the sample size; rather, sample size was limited by the availability of prenatal tissues. Signal intensity measurement details are described in the Immunofluorescence section. For plotting and statistical analysis of scRNA-seq datasets and immune-RNA FISH quantifications, ggplot2 and ggplot2 R packages were used.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The scRNA-seq data of prenatal tissues reported in this paper are available under the following accession numbers: GSE143380 (female cell data) and GSE143556 (male cell data). scRNA-seq datasets are also available online for interactive exploration (http://germline.mcbol.uclal.edu). Previously published RNA-seq data of male and female hPGCs and somatic cells 22,45,46 and single-cell RNA-seq data from female germ cells 44 and from female FCGs 46 that were realanalysed here are available under the following accession codes: GSE63392 (ref. 37), GSE60138 (ref. 37), GSE93126 (ref. 37), GSE79280 (ref. 37) and GSE86146 (ref. 37), respectively. Human conceptus tissue requests can be made to bdsl@u.washington.edu. All other data supporting the findings of this study are available from the corresponding authors on reasonable request. Source data are provided with this paper.

Code availability

Custom scripts used for aligning population RNA-seq, scRNA-seq, data processing and plotting are available on request.

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Author contributions

T.C., P.K. and A.C. designed the experiments. T.C. and R.C. conducted immuno-RNA FISH experiments on tissues and in vitro PSC-derived cells. T.C., R.K. and E.P. generated female hPSC lines from fibroblasts and conducted human blastocyst experiments. D.C. contributed to hPGCLC differentiation experiments. F.-M.H. created an interactive web-site to explore our scRNA-seq datasets. S.L. and K.S.-L. provided the human fetal tissue. T.C. performed scRNA-seq experiments, and the resulting data were analysed by T.C. and L.D.; T.C. analysed bulk RNA-seq data. T.C., K.P. and A.C. interpreted all data and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Female hPGCs from week 4 pf ovaries have lost the H3K27me3 nuclear accumulation. **a**, Distribution of single nucleotide polymorphisms (SNPs) from maternally inherited (x-axis) and paternally inherited (y-axis) alleles in gene expression data of female hPGCs. Each dot represents sum of all detected SNPs per cell for genes on chromosome 1 (Chr1), X-linked genes subject to XCI and escapees of XCI, respectively, based on published scRNA-seq data30. **b**, Representative immunofluorescence staining of OCT4 (magenta), H3K27me3 (green), DAZL (grey) and DAPI (blue) on female hPGCs at week 4 pf prior to gonad formation, when hPGCs are migrating through the aorta-gonad-mesonephros (AGM) (1 sample was analyzed). Insets show a rare OCT4+/DAZL+ cell with no nuclear accumulation of H3K27me3 (inset 1) and an OCT4+/DAZL- cell with H3K27me3 accumulation (inset 2) along the genital ridge of the AGM. Scale bar upper panel 50 microns, lower panel 30 microns. **c**, Percentage of OCT4+/DAZL+ and OCT4+/DAZL- cells with an Xi-like nuclear accumulation of H3K27me3 from the experiment shown in (b); (n=58 cells from 1 AGM). **d**, Quantification of the proportion of DAZL+ female hPGCs at weeks 4, 7 and 12 pf with an Xi-like nuclear accumulation of H3K27me3 (n=50-100 cells per sample in 2 replicates). **e**, Representative immunofluorescence staining of a fetal ovary at week 12 pf with DAZL (magenta), H3K27me3 (green), OCT4 (grey) and DAPI (blue). Inset shows a DAZL+/OCT4 negative female germ cell that is negative for H3K27me3 (1 pair of ovaries were analyzed), scale bar 50 microns. Statistical source data are provided in Source Data Extended Data Fig. 1.
Extended Data Fig. 2 | The X/A ratio is higher in female germ cells than in male germ cells. a, Boxplots presenting the X/A ratio, calculated from the sum of X-linked linked gene expression and the sum of autosomal gene expression, of individual female (red) or male (cyan) germ cells (left panel) and surrounding somatic cells (right panel) obtained from gonads harvested from indicated developmental timepoints (week). b, As in (a), except that the X/A ratio was determined from the mean expression levels of X-linked and autosomal genes per cell. c, Boxplot showing the distribution of the sum of all autosomal (left) and X-linked (right) gene expression, respectively, in individual female and male germ cells across for developmental time points shown in (a). d, As in (a), except that the X/A ratios in female and male FGCs across developmental time from a published study are shown40. e, X/A ratio per single cell in female and male gonadal somatic cells from all developmental ages accompanying the data shown in (d). Wilcoxon statistical testing between age matched samples, NS- Not Significant, * p<0.05, ** p<0.01, *** p<0.001. a-c: n = 49528 cells analyzed across 10 independent experiments and d-e: 1016 cells analyzed from published dataset40 in total.
Extended Data Fig. 3 | Female hPGCs dampen X linked gene expression before entering meiosis. a, Boxplots of the X/A ratios of female germ cells along the developmental trajectory and in female gonadal somatic cells, as described in Fig. 5, except that the top 5% highest expressed genes were excluded from the analysis. b, As in (a), except that the bottom 5% of expressed genes were excluded from the analysis. c, Boxplots of the X/A ratios in female germ cells and female gonadal cells as described in Fig. 5, except that the X/A ratios were calculated from the mean value of X-linked and autosomal gene expression per cell. d, Sum of all autosomal gene expression normalized counts in female germ cells organized by clusters along the developmental trajectory. e, Sum of all X-linked gene expression in female germ cells organized by clusters along the developmental trajectory. X-linked gene expression increases in clusters 7-9 coincident with entrance into meiosis and repression of the naïve-like pluripotency program. f, Germline trajectory analysis of previously published scRNA-seq data from female FCGs. The hPGCs state with pluripotency program expression is captured with clusters 0-4, and meiotic entry in cluster 5-7. g, Boxplots of the X/A ratios for female germ cells and female gonadal somatic cells for the data set shown in (f). From cluster 5 onwards, X/A ratios in differentiating female germ cells are higher than gonadal somatic cells. Wilcoxon statistical testing for (a), (b), (c), (g). NS= Not Significant, * p<0.05, ** p<0.01, *** p<0.001.
Extended Data Fig. 4 | See next page for caption.
**Extended Data Fig. 4** | Male hPGCs do not change X/A ratio upon sex specific differentiation. 

**a.** Male germ cells from the scRNA-seq data shown in Fig. 4a–c were projected along the developmental trajectory, and five clusters (0–4) were identified (n = 282 cells pulled from 5 samples). The pluripotency program is repressed in cluster 4, coincident with increased expression of prospermatogonia genes and exit from the cell cycle. 

**b.** Expression of marker genes along the developmental trajectory of male germ cells defined in (a). 

**c.** Box plots showing that X/A ratios in male germ cells along the developmental trajectory and in surrounding male somatic cells. 

**d.** As in (c), except that the top 5% highest expressed genes were excluded from the analysis. 

**e.** As in (c), except that bottom 5% of genes were excluded. 

**f.** As in (c), except that the top and bottom 5% of expressed genes were excluded. 

In total n = 24740 cells analyzed across 5 independent experiments in c–f. 

**g.** Sum of all autosomal gene expression per cell in male germ cells along the developmental trajectory, showing no dramatic differences across the clusters. 

**h.** As in (g), except for X-linked gene expression, showing no dramatic differences across the clusters. 

**i.** Germine trajectory analysis for male FGCs, identified 7 clusters (0–6). Marker gene expression is given for these clusters. 

**j.** Box plots of the X/A ratios in male FCGs along the developmental trajectory defined in (i), showing an increase in cluster 2 relative to cluster 1. In total, n = 779 cells analyzed from published dataset in j. Wilcoxon statistical testing used for (c–f) and (j). NS- Not Significant, * p<0.05, ** p<0.01, *** p<0.001.
Extended Data Fig. 5 | XIST expression correlates with the X/A ratio. a, Boxplot depicting the expression of XIST in female germ cells organized by clusters along the developmental trajectory defined for the published FCG data set in Extended Data Fig. 3f,g. XIST expression is significantly reduced from cluster 5 onwards. b, XIST expression in mature oocytes and granulosa cells from scRNA-seq data of adult ovary. c, Scatter plot of average XIST expression (y-axis) and average X/A ratio (x-axis) for female germ cells clusters 6-9 (from Fig. 4d), capturing the entrance into meiosis. d, X/A ratios in female hPGCs and meiotic germ cells clustered based on expression of lncRNA XIST, ZP3+ primordial oocytes are clustered separately. e, Boxplots depicting XIST expression in male germ cells ordered along the developmental trajectory defined in Extended Data Fig. 4a–h, indicating that XIST transcripts are rarely detected in male germ cells. f, As in (e), except for male FGCs from Extended Data Fig. 4i,j. g, Average XACT cloud size in week 8 and 14 pf hPGCs with different patterns of XIST expression, error bars show standard deviation of the cloud sizes (76 cells analyzed in total from 2 independent experiments). Wilcoxon statistical testing used for (a), (d), (f), (g). NS- Not Significant, * p<0.05, ** p<0.01, *** p<0.001. Number of cells analyzed across 5 independent experiments: a. n = 1016 cells, b. n = 148 cells, d. n = 1938 cells, f. n = 779 cells.
Extended Data Fig. 6 | XACT marks male and female hPGCLCs in vitro. Summary of the hPSC differentiation figure shown in Fig. 2. Due to XCI erosion, XACT is expressed from the Xa and the eroded X-chromosome in primed, female human pluripotent stem cells. The Xe state is transmitted into differentiated cells and upon hPGCLC differentiation. Moreover, XACT is maintained in hPGCLCs, whereas somatic cells silence XACT. Consequently, female hPSC-derived PGCLCs carry two XACT clouds and male hPSC-derived PGCLCs one.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

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☐ A description of all covariates tested

☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection Custom scripts used for aligning population RNA-seq, scRNA-seq, data processing and plotting are available upon request

Data analysis For scRNA-seq data analysis Scanpy v1.4.7, louvain 0.6.1. Cellranger v2.2. FACs sorting data was analyzed with FlowJo v10. R packages: ggplot2 v2.2.3, reshape v0.8.8, biomaRt v2.38.0

For manuscripts utilizing custom algorithms or software that are not central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The accession number for the scRNA-seq data of prenatal tissues reported in this paper are GSE143380 (female cell data) and GSE143356 (male cell data). scRNA-seq data sets are also available for interactive exploration at http://germline.mcdb.ucla.edu.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample size was determined by availability of human prenatal tissues. |
| Data exclusions | No data was excluded, all biological samples received for this study were used either for scRNA-seq or immuno-RNA FISH experiments, no . Following scRNA-seq, genes expressed in less than five cells were discarded, and cells with less than 250 detected genes were filtered out. |
| Replication | All experiments on individual biological samples were performed in technical replicates. Human prenatal gonadal samples were each considered a single replicate and where possible results in scRNA-seq were verified by immuno-RNA FISH. |
| Randomization | This study did not involve comparison between experimental and control groups. For immuno-RNA FISH experiments random sections of the tissue were selected for staining |
| Blinding | This study did not use blinding for data analysis as the prenatal gonadal samples sent to us were already identified as male or female. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
| n/a | Involved in the study |
| ☒ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☐ | Palaeontology and archaeology |
| ☒ | Animals and other organisms |
| ☒ | Human research participants |
| ☒ | Clinical data |
| ☐ | Dual use research of concern |
| n/a | Involved in the study |
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

Antibodies

| Antibodies used | Antibodies used Rabbit anti-human NANOG Abcam Cat#ab109250 1/200; Goat anti-human OCT4 Santa Cruz Cat#sc-8628 1/200; Rabbit anti-human H3K27me3 Millipore Cat#07-449 1/400; Mouse anti-human DAZL Santa Cruz Cat#sc-390929 1/400; BV421 conjugated anti-human/mouse CD49f (ITGA6) BioLegend Cat#313624 1/60; Alexa Fluor 488-conjugated anti-human CD326 (EPCAM) BioLegend Cat#324210 1/60. |
| Validation | NANOG antibody was validated the manufacturer, in human seminoma tissues by IHC and as Negative control PBS was used as a primary antibody. OCT4 antibody was published before in Chen et al. BOR 2017. H3K27me3 antibody was published before in Gell et al. Stem Cell Research 2018. DAZL antibody was published before in Kuo, P.L., et al. Fertil. Steril. 2004. ITGA6 and EPCAM antibodies were published in Chen et al. BOR 2017 |

Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | Cell line source(s) UCLA2 (46, XY) hESCs lines were generated at UCLA from single blastocyst and published in Diaz et al., Human Molecular Genetics 2012. Female MZT04 hIPSC (46, XX) was generated by reprogramming human fibroblasts into iPSCs using Sendai virus Reprogramming (CytoTune) and published in Pandolfi et al., Stem Cell Research 2019. |
| Authentication | Authentication UCLA2 was authenticated by SNP/CNV analysis, MZT04 was authenticated using Short tandem repeat analysis to the parental fibroblasts. |
Mycoplasm contamination | All lines were routinely tested for mycoplasm contamination and tested negative

Commonly misidentified lines (See ICLAC register) | No commonly misidentified lines were used in this study

**Human research participants**

**Policy information about studies involving human research participants**

**Population characteristics** The human subjects research portion of this project included pre-implantation embryo donors and fetal tissue. The pre-implantation embryo donors were couples who had sought treatment for infertility at an in vitro fertilization clinic. As a result of this treatment, the donors generated surplus embryos that were frozen. For the fetal tissues, donors were women of reproductive age who were seeking an elected termination of pregnancy.

**Recruitment** Recruitment of embryo donors involved sending a UCLA IRB approved flyer to collaborating IVF laboratories. The laboratories provided the flyer to patients with frozen surplus embryos. The flyer contained details on the project, and UCLA contact details for additional information. Patients interested in donating embryos would then contact UCLA to be recruited into the study. No payments were made to clinics or donors for surplus embryos.

**Ethics oversight** Use of human pre-implantation embryos in this research project followed California State law and the 2016 ISSCR guidelines for Stem Cell Research. Specifically, this involved annual review and approval by the UCLA Institutional Review Board (IRB) and the human embryonic stem cell research oversight committee (ESCR). Together, these committees approve the process of informed consent, and experiments using human embryos for research purposes. Patients were not paid for participation, and all donors were informed that the embryos would be destroyed as part of the research study. For fetal tissue samples provided by the University of Washington, regulatory oversight involved the University of Washington IRB approved Human Subjects protocol, combined with a Certificate of Confidentiality from the Federal Government. For fetal samples received from the University of Tübingen, the research project was also approved by the research ethics committee of the University of Tübingen. No payment was made to fetal tissue donors, and all patient identifiers were removed before shipping to UCLA.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

**Flow Cytometry**

**Plots**

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

**Methodology**

**Sample preparation** hPGCLC aggregates were dissociated 0.05% Trypsin-EDTA (GIBCO, 25300-054) for 10 min at 37°C. The dissociated cells were stained with conjugated antibodies, washed with FACS buffer (1% BSA in PBS) and resuspended in FACS buffer with 7-AAD (BD Pharmingen, 559925) as viability dye. The conjugated antibodies used in this study include ITGA6 conjugated with BV421 (BioLegend, 313624, 1:60), EPCAM conjugated with 488 (BioLegend, 324210, 1:60). The single cell suspension was sorted for further experiments using BD FACSAnA fluorescence-activated cell sorting machine. FACS data was analyzed with FlowJo v10 software. Double positive cells for ITGA6 and EPCAM (hPGCLCs) and negative cells (non-hPGCLCs) were collected in hPGCLC media and plated on human plasma fibronectin coated coverslips overnight. Following morning, RNA FISH was performed using the coverslips.

**Instrument** BD FACSAria

**Software** Manufacturers software was used for sorting. Data analysis was done using FlowJo v10

**Cell population abundance** Double positive hPGCLC population was sorted using EPCAM and ITGA6. Abundance of cell population indicated in the figure. All somatic cells were selected outside hPGCLC gate

**Gating strategy** Gating was done by single color staining of hESCs. Complete strategy is provided in the supplementary information

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.