Running title: Epigenetic reprogramming in human primordial germ cells

TITLE: Characterisation of the epigenetic changes during human gonadal primordial germ cells reprogramming

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ABSTRACT

Epigenetic reprogramming is a central component of the mammalian germline development. Genome-wide DNA demethylation observed in primordial germ cells (PGCs) is a prerequisite for erasure of epigenetic memory, preventing the transmission of epimutations to the next generation. Apart from DNA demethylation, germline reprogramming has been shown to entail reprogramming of histone marks and chromatin remodelling. Contrary to other animal models, there is limited information about the epigenetic dynamics during early germ cell development in humans. Here we provide further characterization of the epigenetic configuration of the early human gonadal PGCs. We show that early gonadal human PGCs are DNA hypomethylated and their chromatin is characterized by low H3K9me2 and high H3K27me3 marks. Similarly to the previous observation in the mouse, human gonadal PGCs undergo dynamic chromatin changes concomitant with the erasure of genomic imprints. Interestingly, and contrary to mouse early germ cells, expression of BLIMP1/PRDM1 persists in all gestational stages in human gonadal PGC and is associated with nuclear LSD1. Our work provides important additional information regarding the chromatin changes associated with human PGCs development between 6 and 13 weeks of gestation in male and female gonads.

Introduction

Primordial germ cells (PGCs) derived from epiblast of the pre-gastrulating embryos are the founder population of the future gametes. A unique characteristic of PGCs is the ability to acquire totipotency to give rise to the next generation. This ability is crucially underpinned by an existence of an extensive epigenetic reprogramming, that is a hallmark of PGC development in mammalian species [1,2]. Genome-wide DNA demethylation in mouse PGC results in the complete erasure of methylation marks in single-copy and imprinted genes, and a moderate reduction of methylation levels of retrotransposons and other repetitive elements [3,4]. This germline demethylation is critical for erasing epigenetic memory and preventing the transmissions of epimutations to the next generation [3,5]. Apart from dynamic changes in DNA methylation, early mouse germ cells are characterised by distinctive chromatin signature [1]. Reduction in the heterochromatin H3K9me2 mark observed in nascent mouse PGCs is followed by an increase in repressive H3K27me3 in migrating mouse germ cells between E7.5 and E8.5, at the time when these cells undergo G2 arrest and transcriptional quiescence [6]. Following their migration and the entry into the genital ridges, mouse PGCs undergo erasure of genomic imprints followed by major conformational changes in chromatin including loss of linker histone H1 and the replacement of nucleosomal histones [7,8]. Together, these dynamic events define a critical period for the epigenetic reprogramming in the mouse germ line [3,6,7].

Considerable progress has been made regarding our understanding of the molecular regulation of the mouse developing germ line in recent years. In particular, the establishment of the germline fate has been attributed to the combination of three key

transcription factors: Blimp1/Prdm1, Prdm14 and Tfap2cAp2gamma [9,10] and the key transcriptional changes associated with the early mouse PGC development have been described. Interestingly, although it has been shown that cells recapitulating early mouse PGC development (PGC like cells, PGClCs) can be derived *in vitro* from cultured pluripotent mouse ESCs, these cells resemble migratory mouse PGCs and require transplantation into the gonadal environment to undergo the epigenetic changes (including erasure of genomic imprints) normally associated with the gonadal germline reprogramming [1,11]. These observations clearly advocate the need for better understanding of the gonadal germline reprogramming process on the molecular level.

Most of our knowledge regarding mammalian germline development originates from studies in mice. Only limited knowledge is available regarding the sequence of events during PGC development in other species; studying these events in non-rodents is thus important to establish whether conserved mechanisms underlie PGC development in mammals. In this context, porcine model has been considered as a suitable model to study mammalian development [12,13], due to the developmental and physiological similarities with most other mammals, including humans. Recent studies of porcine PGCs demonstrated that, similarly to previous observation in the mouse, reprogramming of H3K9me2 and H3K27me3 histone marks precedes also germline DNA demethylation in pig, suggesting that hallmarks of germline epigenetic reprogramming might be conserved [14,15]. Nonetheless, limited information is available regarding these epigenetic events in human.

The presented study focuses on the investigation of the gonadal epigenetic reprogramming in the human germ line. Our results document that early human gonadal

PGCs are DNA hypomethylated and share the distinct chromatin configuration previously observed in PGCs in the mouse and pig. Using bisulphite sequencing of the isolated human gonadal PGCs we furthermore show, that the imprinted H19 DMR undergoes methylation erasure around week 11 of gestation. This is accompanied by loss of H3K27me3 and dynamic gain in transcriptionally permissive histone modifications. Our results thus draw parallels between the chromatin changes observed in human gonadal germ cells and the previously described observations in the mouse and pig PGCs. Interestingly, although in the mouse Blimp1/Prdm1 expression is lost in PGCs soon following their entry into the genital ridge, human PGCs retain BLIMP1/PRDM1 expression in all gestational stages analysed (up to 12 weeks of gestation). Expression of this germline determinant is also not associated with nuclear PRMT5 as previously observed in the mouse [16], but is concomitant with the nuclear LSD1, a histone demethylase previously shown to associate with BLIMP1/PRDM1 in human plasma cells [17].

Collectively, our work provides new information about human PGCs reprogramming *in vivo* aiding to our efforts towards understanding of the cellular and epigenetic mechanisms leading to and governing human totipotency.

Materials and Methods

Human fetal sample collection

Human fetal samples from 6 to 13 weeks postconception (wpc) were used. Oral and written information was given and informed consent was obtained from all women, according to and approved by The Spanish and Catalan Committee on Biomedical Research Ethics (Nº:1811521). Calculation of gestational age was based on the information about the last menstrual period, and measurements of crown rump and foot lengths. The sex of all samples was confirmed by performing genomic PCR for human gender determination according to US patent (US007432362B2) (Suppl Fig 1).

Immunofluorescence microscopy

The specimens were fixed in 4% paraformaldehyde, dehydrated with graded alcohols, cleared in xylene and embedded in paraffin wax. Serial sections (5µm) were de-paraffinized, rehydrated and antigen retrieval (pH:9) was performed. The immunofluorescence staining was performed as previously described [18] The following antibodies and their dilutions were used: OCT-3/4 (sc-5279 and sc-8628, SantaCruz, 1:25), NANOG (AF1997, R&D systems, 1:25), SSEA-1 (MC-480, Developmental Studies Hybridome Bank at University of Iowa, 1:1), cKIT/CD117 (A4502, Dako, 1:100), VASA (AF2030, R&D systems, 1:20), H3K4me1 (ab8895, Abcam, 1:50), H3K9ac (ab10812, Abcam, 1:50), H3K27me3 (07-449, Millipore, 1:50), H3K4me3 (07-473, Millipore, 1:50), H3K9me2 (07-441, Millipore, 1:50), H3K9me3 (07-442, Millipore, 1:50), LSD-1 (ab17721, Abcam, 1:50), DHX38 (10098-2-AP, ProteinTech Group, 1:50), PRMT5 (sc-22132, SantaCruz, 1:25), BLIMP1 (9115S, Cell Signalling, 1:100) . The Alexa Fluor Series from Invitrogen were used as secondary

antibodies (all 1:200). Images were taken using a Leica SP5 confocal microscope. Quantitative analyses were performed by counting cKIT/OCT4 and VASA/OCT4. Data are expressed as mean ± SD (Suppl Fig 2).

Quantification of staining intensity by confocal microscopy

The quantification of staining intensity was performed using LAS AF Leica Software. Fluorescence was detected using same confocal settings. Mean fluorescence intensity/area values were measured for 30 nuclei germ cells and surrounding somatic cells. To normalize between different samples, the intensity of each histone mark staining was calculated as the mean pixel intensity in every PGC divided by the mean pixel intensity in surrounding somatic cells [7] (Suppl Fig 7). The error bars represents standard deviations. Statistics of immunofluorescence staining data according to T-tests significance [$p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)] (Fig 1, Fig 2, Fig 3, Fig 4, Fig 6, Suppl Fig 3 and Suppl Fig 4).

5meC staining

Sections were dewaxed, rehydrated and antigen retrieval (pH:6) were performed. In order to block non-specific binding, alkaline phosphatase and endogenous peroxidase activity, sections were first blocked with 3% donkey serum, secondly were incubated with levamisole and finally treated with 2% H2O2. All sections were incubated with VASA (AF2030, R&D systems, 1:25) and 5meC (16233D3, Calbiochem, 1:50) antibodies. For detecting VASA antibody, sections were incubated with a secondary antibody alkaline phosphatase conjugated (705-055-147, Jackson Lab., 1:100). Finally sections were revealed by using EnVisionTM System, BCIP/NBT (Dako) and DAB.

Isolation of human PGCs

Human fetal gonads were dissociated in 0.25% trypsin-EDTA (Gibco) at 37º C for 20 min and collected by centrifugation at 2000 rpm in a microcentrifuge. Dissasociated cells were resuspended in a buffer containing 1% FBS (Gibco). cKit+/CD117+ cells were selected by using an immunomagnetic separation system (MiniMACS, 130-091-332, Miltenyi Biotec). Purification efficiency (higher than 90%) was verified by alkaline phosphatase staining (SK-5100, Vector Laboratories) (Suppl Fig 1).

Promoter methylation analysis

The isolated hPGCs and their gonadal somatic cells were embedded in agarose, lysed and the chromosomal DNA subjected to the bisulphite treatment [19]. The promoter sequences for H19 were amplified by a nested PCR with two subsequent reactions using primers and conditions previously described [20,21]. The resulting amplified products were cloned into pGEM T Easy plasmids, amplified in TOP10 chemically competent cells, purified and sequenced. Plasmid purification and sequencing were performed for at least 12-20 positive clones. To analyse the data, mutagenesis rates of > 95%, excluding putative methylation sites, were considered acceptable to proceed. Sequences were aligned to the reference sequence by Quantification tool for methylation analysis (QUMA). Percentage of methylation was calculated according to the number of conserved CpG islands related to the total CpG islands analysed.

Results

The dynamics of cKIT, VASA, OCT4 and NANOG expression in human fetal gonads.

Following their migration, human PGCs have been shown to colonize the fetal gonads between week 6 and 8 of gestation [22].To follow epigenetic changes occurring during human early gonadal PGC development, we have collected and analysed 90 samples of human fetal gonads between week 6 and 13 of gestation. We first carried out assessment of the expression of cKIT, VASA, OCT4 and NANOG in human fetal gonads (male and female) between 6 to 12 weeks of gestation by immunofluorescence. We observed that cKIT, VASA, OCT4 and NANOG were present in PGCs of all stages studied in both sexes. Expression of these markers was restricted to germ line, as expected. Although all human PGCs were expressing cKIT across all analysed stages (see also [23]) early gonadal PGCs showed heterogeneity in VASA staining (gestation week 6-9) (see asterisks in Fig 1, Fig 5, Fig 6 and Suppl. Fig 5). Additionally, starting from gestation week 11 we observed presence of PGCs that were VASA+/cKIT+ but OCT4-/NANOG- (see arrows in Suppl Fig 2 and arrowheads in Fig 1). We note that this timing coincides with the reported initiation of meiosis in human female PGCs [18] (Suppl Fig 2). In this context mouse gonadal PGCs have been shown to downregulate Oct4 expression upon entry into meiosis [24].

Unique germline epigenetic signature is conserved across species

Chromatin features of the mouse early germ cells have been extensively studied [5,7]. In the mouse, early PGCs are characterized by a genome-wide loss of H3K9me2, a facultative heterochromatin mark, followed by global increase in the polycomb H3K27me3 histone modification [5,7]. Interestingly, the same chromatin configuration is recapitulated in *in vitro* mouse derived PGC like cells (PGCLCs) [11,25]. In order to

understand whether this specific germline chromatin configuration is conserved also in human we stained human fetal gonads cells with specific antibodies recognizing these chromatin marks. Consistent with findings in the mouse, both male and female human PGCs stained weakly or below the level of detection for H3K9me2 in comparison with somatic cells across all gestational stages analysed (Fig. 1; Suppl Fig 7). In addition, early gonadal PGCs (gestation week 6-8 in the male and 6-7 in the female) were characterized by high level of H3K27me3 in comparison to the surrounding somatic cells of the fetal gonad (Fig. 2; Suppl Fig 7). This confirms that the chromatin germline signature observed in the mouse might be conserved across species and thus represents a unique germline attribute.

Although low H3K9me2 persists in the pre-meiotic mouse PGCs, H3K27me3 observed in the migratory and early postmigratory PGCs disappears around the time of imprint erasure (E11.5 in the mouse, [7]). Strikingly, and in agreement with the previous observations [23], H3K27me3 diminishes also in human PGCs of both sexes after 9 weeks of gestation in (Fig. 2; Suppl Fig 7).

Chromatin modifications, permissive and repressive marks

To provide further characterization of the global chromatin dynamics during human PGC development, PGCs and somatic cells were stained with specific antibodies against the transcriptionally permissive marks: H3K4me1, H3K4me3 and H3K9ac. Male and female germ cells stained strongly for H3K4me1 and H3K4me3 in comparison with the surrounding somatic cells in most of gestational stages analyzed (Suppl Fig. 3; Suppl Fig 7 and Fig. 3). Although H3K9ac was also generally higher in PGCs than in the surrounding somatic cells, we observed a clear peak of signal from 10 to 13 weeks of gestation in both sexes (Fig. 4; Suppl Fig 7).

Contrary to general enrichment of transcriptionally permissive chromatin marks, we observed lower H3K9me3 staining in male germ cells in comparison with the surrounding somatic cells, (Suppl Fig. 4 and Suppl Fig 7). This was not apparent in the female samples.

Methylation status of human gonadal PGCs

Dominant feature of the germline chromatin is the global erasure of DNA methylation (5meC) [1,6,26]. In the mouse, although decrease in global 5mC levels is detectable following the establishment of the low H3K9me2/ high H3K27me3 global chromatin signature in migrating PGCs [5], the complete removal of genome-wide DNA methylation including the erasure of genomic imprints proceeds only once the mouse PGCs colonized the genital ridges of the midgestation mouse embryo at E11.5 [4,7].

In order to understand the general methylation status of human PGCs we carried out 5-methyl cytosine (5meC) staining in combination with a germ cell specific marker, VASA. Our results indicate that the 5meC levels detected in human gonadal PGCs were lower than those found in the surrounding gonadal somatic cells. We also note that this became particularly apparent from gestation week 10 (Suppl Fig 1).

We next evaluated the DNA methylation status of the promoter of the maternally expressed, paternally imprinted gene H19. First, human PGCs were isolated by MACS at different gestational stages using magnetic beads coupled to c-KIT antibody. The purity of the isolated PGCs was confirmed (90-95%) by Alkaline Phosphatase staining (Suppl Fig 1). Bisulphite sequencing analysis revealed that in the PGCs of both genders, H19 DMR shows an apparent loss of methylation starting from gestation week 9. In male PGCs we detected a decrease from 80% to 29%; in female PGCs we detected a decrease from 60% to 20% (Suppl Fig 1). The loss of methylation at the H19 DMR

corresponds with the timing of the pronounced global DNA hypomethylation observed in the human gonadal PGCs by 5mC at gestation week 10 as discussed above (Suppl Fig 1). Concomitantly all these changes coincide with the timing of the observed chromatin changes (loss of H3K27me3 – Fig 2 and upregulation of H3K9ac – Fig 4).

Blimp1-Prmt5 complex

Data from murine model indicates the important role of BLIMP/PRMT5 complex and his target DHX38 during the specification of the mouse PGCs within the epigenetic program. In order to ascertain whether the expression of BLIMP1/PRMT5 complex follows a similar pattern to the one described in mouse we evaluated the presence of this complex during human PGCs development. Although we confirmed germline specific expression of BLIMP and high expression levels of PRMT5 in human PGCs, we consistently found that the expression of BLIMP1 was restricted to the nucleus whereas PRMT5 expression was mainly localized in the cytoplasm of PGCs in both sexes across all gestational stages examined (Fig 5 and Suppl Fig 5).

Dhx38 helicase has been previously shown to be one of the targets repressed by the Blimp1/Prmt5 complex in the mouse PGCs [16]. In agreement with the apparent lack of the BLIMP/PRMT5 complex formation in the human gonadal PGCs, DHX38 was found in both nucleus and cytoplasm of all analysed human PGCs independently of gestational stage and sex (Suppl Fig.6).

It has been previously shown that during plasma cell differentiation BLIMP1 directly interacts with the histone lysine demethylase, LSD1 [17]. Considering the lack of evidence for the BLIMP/PRMT5 complex formation in the human gonadal PGCs it is of interest that we detected high amount of LSD1 protein in male and female PGCs in

comparison with the somatic cells in all analysed gestational stages (Fig. 6; Suppl. Fig 7).

Discussion

The presented study provides characterization of epigenetic changes in early human gonadal PGCs from 6 to 13 weeks of gestation. We evaluated the epigenetic status of human PGCs by assessing changes in global chromatin marks, the methylation status of H19 imprinted gene and changes associated to methylation status genome-wide.

We first characterized the expression of markers associated with stem cells and pluripotency during the early gonadal stages of human PGC development. In agreement with previous studies [14,23,24,27,28,29] we show that gonadal PGCs are characterized by cKIT expression. Although it has been previously reported that human gonadal PGCs are expressing SSEA-1 [30, 31], the expression of this marker is not restricted to PGCs in human fetal gonads (Suppl Fig 2C). In agreement with a recent publication [28], we observed that majority of PGCs were VASA positive at 6 weeks of gestation, however all human PGCs were VASA positive by 9 weeks of gestation. This is in agreement with the mouse model, where Vasa (mvh) expression becomes prominent only once mouse PGCs colonize the genital ridges [32]. All early human gonadal PGCs express OCT4 and NANOG, but the proportion of OCT4 and NANOG positive germ cells declines from gestation week 9 onwards, with the fraction of cKIT +/OCT4- PGCs increasing up to 25% in late stages (w12-w13). Our data are thus in agreement with the recently published observations [28]. The absence of expression of OCT4 in female PGC samples coincides with the reported entry of human PGCs into meiosis [22,31] as also previously observed in the mouse [24].

In the mouse, pre-migratory PGCs initiate a process of reprogramming that erases epigenetic marks. One of the key epigenetic changes in pre-migratory and early migratory mouse PGCs is a loss of H3K9me2 that is closely followed by an accumulation of H3K27me3 signal [5,7,25]. Interestingly, our stainings of early human gonadal PGCs confirm the presence of analogous chromatin changes also in the human early developing germ line (Fig 1 and 2) [27,28,29]. As similar changes in global chromatin configuration has been also reported in porcine PGCs, our findings clearly document that unique chromatin features of early germ line are conserved, at least within mammalian clade.

Following their entry into the genital ridges, mouse PGCs undergo erasure of genomic imprints and dynamic changes in chromatin modifications [7]. In analogy to the mouse model and in agreement with recent publications [23,27,28,29], our results show that human gonadal PGCs lose H3K27me3 following week 9 of gestation. Again, similar observation has been previously made using porcine model further strengthening the notion of the existence of the conserved mechanism underlying germline epigenetic reprogramming [15].

Interestingly, also in the human gonadal PGCs, the timing of the H3K27me3 loss overlaps with the observed reduction of DNA methylation at imprinted H19 DMR and also with a clear global hypomethylated state as observed by global 5mC staining (Suppl Fig 1). This finding yet again parallels similar observations in the mouse, pig and human models [7,14,15,27,28,29]. We also notice, that the presence of the early germline chromatin signature (low H3K9me2, H3K27me3) *prior* to the erasure of genomic imprints defines a distinct developmental window with high propensity of

PGCs to give rise to pluripotent embryonic germ (EG) cells in both mouse and human [30,31,32,33,34,35,36].

Finally, Blimp1, a transcriptional repressor, has been described as one of the key determinants of the mouse germ line [10,16]. In the mouse migratory and early post-migratory PGCs, Blimp1 has been reported to form a repressive regulatory complex with Prmt5, a type II arginine specific histone methyltransferase [16]. Although a previous report suggested a possible existence of a similar regulatory complex also during human PGC development [27,29,37], our data clearly show that BLIMP1 expression is restricted to the nucleus of PGCs in human fetal gonads (Fig 5), while PRMT5 shows cytoplasmic localization in human germ cells across all gonadal stages analysed (Suppl Fig 5). In addition, DHX38, a target of repressive Blimp1/Prmt5 complex in the mouse PGCs [16], was found to be expressed in all stages of human PGCs analysed in our study (Suppl Fig 6).

Although our observations argue against the presence of the nuclear BLIMP1/PRMT5 complex in early gonadal human PGCs, we note that nuclear BLIMP1 coincides in human PGCs with high levels of Lysine-specific demethylase-1 (LSD1) (Fig. 6). In this context, BLIMP1 has been previously shown to interact with LSD1 during human plasma cell differentiation [17]. Additionally, LSD1 has been implicated in gene silencing in *Drosophila* PGCs and also during germline reprogramming in *C.elegans* [38,39,40]. Further biochemical and functional studies will thus be required to confirm the functional relevance of this intriguing observation.

In summary our study provides further characterization of the epigenetic changes observed in early human gonadal PGCs (Fig 7) and thus complements the recently published genome-wide transcriptional and methylation studies [27,28,29]. Although the relative timing might differ due to species specific developmental progression, our data show that key epigenetic changes observed in the germ line are conserved between mouse, pig and human. Our work further demonstrates that gonadal epigenetic reprogramming starts in human PGCs around 9 weeks of gestation (Fig 7), which is in agreement with the recently published observations [27,28,29]. Last, but not least, our observations regarding BLIMP/PRMT5 complex highlight potential differences between germline regulatory complexes in mouse and human and suggest a potential role for LSD1 as a new epigenetic regulator during gonadal reprogramming in humans.

Conclusion

In summary our work provides new information about human PGCs reprogramming in vivo aiding to our efforts towards understanding of the cellular and epigenetic mechanisms leading to and governing human totipotency.

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Figure legends

Figure 1. Chromatin changes of H3K9me2 in human fetal PGCs.

A Immunofluorescence of primordial germ cells and somatic cells for detecting H3K9me2 (green), VASA (red), OCT4 (yellow) and DAPI (blue) proteins. Fetal ovaries and testis were stained. Scale bar 10 μm. (*: Heterogeneity in VASA staining; *arrowheads*: VASA+/OCT4- cells).

B Quantification of H3K9me2 signal in human germ and somatic cells. The error bars represent standard deviation. ($p<0.05$ in some stages of fetal testis).

Figure 2. Chromatin changes of H3K27me3 in human fetal PGCs.

A Immunofluorescence of primordial germ cells and somatic cells for detecting H3K27me3 (green), VASA (red), OCT4 (yellow) and DAPI (blue) proteins. Fetal ovaries and testis were stained. Scale bar 10 μm.

B Quantification of H3K27me3 signal in human germ and somatic cells. The error bars represent standard deviation. ($p<0.05$ in a stage of fetal ovaries)

Figure 3. Chromatin changes of H3K4me3 in human fetal PGCs.

A Immunofluorescence of primordial germ cells and somatic cells for detecting H3K4me3 (green), VASA (red), OCT4 (yellow) and DAPI (blue) proteins. Fetal ovaries and testis were stained. Scale bar 10 μm.

B Quantification of H3K4me3 signal in human germ and somatic cells. The error bars represent standard deviation. ($p<0.05$ in a stage of fetal testis; $p<0.01$ in a stage of fetal ovaries).

Figure 4. Chromatin changes of H3K9ac in human fetal PGCs.

A Immunofluorescence of primordial germ cells and somatic cells for detecting H3K9ac (green), VASA (red), OCT4 (yellow) and DAPI (blue) proteins. Fetal ovaries and testis were stained. Scale bar 10 μm.

B Quantification of H3K9ac signal in human germ and somatic cells. The error bars represent standard deviation. ($p<0.05$; $p<0.01$ in some stages of fetal testis and ovaries).

Figure 5. The expression of Blimp1 in human fetal gonads. Immunofluorescence of primordial germ cells and somatic cells for detecting BLIMP1 (green), VASA (red), OCT4 (yellow) and DAPI (blue) proteins. Scale bar 10 μm. (*: Heterogeneity in VASA staining).

A Fetal testis from 7 to 12 weeks of gestation

B Fetal ovaries from 7 to 12 weeks of gestation.

Figure 6. Chromatin changes of LSD1 in human fetal PGCs.

A Immunofluorescence of primordial germ cells and somatic cells for detecting LSD1 (green), VASA (red), OCT4 (yellow) and DAPI (blue) proteins. Fetal ovaries and testis were stained. Scale bar 10 μm. (*: Heterogeneity in VASA staining).

B Quantification of LSD1 signal in human germ and somatic cells. The error bars represent standard deviation. ($p<0.05$ in some stages of fetal testis and ovaries; $p<0.01$ in a stage of fetal ovaries).

Figure 7. Summarized time course of human gonadal epigenetic reprogramming

A Cellular dynamics and gonadal epigenetic reprogramming in human germ cells

B Mouse, Pig and Human epigenetic reprogramming time window in germ line

Supplementary Figure 1. Changes of global DNA methylation and methylation status of H19 gene in human PGCs.

A Primordial germ cells and somatic cells were double stained with VASA antibody (blue) in the cytoplasm and 5meC antibody (brown) in the nucleus.

Immunohistochemistry by using fetal ovaries and testis from 7 to 12 weeks of gestation. Cells in blue are PGCs and cells in brown are somatic cells. Scale bar 10 µm.

B Schematic of expected methylation status of 18 CpG islands in region 2000-2200 of the H19 differentially methylated region (DMR). Each lane of circles represents one individual clone. Open circles represent unmethylated CpGs, and closed circles represent methylated CpGs.

C CpGs in the H19 promoter DMR were analysed by bisulphite sequencing in male PGCs from 7 to 11 weeks of gestation

D CpGs in the H19 promoter DMR were analysed by bisulphite sequencing in female PGCs from 7 to 10 weeks of gestation

E Control of somatic cell from gonads

F Sexing PCR. To determine the sex of the fetal gonads we performed a genomic PCR. M: male; F: female; CM: control male and CM: control female.

G Alkaline phosphatase staining. To determine the purity of the cKIT+ immunomagnetic separation technique (MiniMACs) we performed the tissue non-specific alkaline phosphatase staining, (a) cKIT positive fraction and (b) cKIT negative fraction.

Supplementary Figure 2. Characterization of the human fetal gonads.

A Immunofluorescence of primordial germ cells and somatic cells for detecting OCT4 (green), cKIT (red), NANOG (yellow) and DAPI (blue) proteins. Fetal ovaries from 6 to 12 weeks of gestation and fetal testis from 7 to 12 weeks of gestation were stained. Scale bar 25 µm. (*Arrows*: cKIT+/OCT4-/NANOG- cells).

B The dynamics of cKIT, OCT4 and VASA expression in the fetal gonad. Quantification cKIT+, OCT4+, VASA+, cKIT+/OCT4+ and VASA+/OCT4+ in fetal ovaries and testis

C Immunofluorescence of primordial germ cells and somatic cells for detecting SSEA1 (red), NANOG (green) and DAPI (blue) proteins. Fetal testis from 14 weeks of gestation were stained. Scale bar 50 μm. (*Arrowheads*: SSEA1+/NANOG- cells).

Supplementary Figure 3. Chromatin changes of H3K4me1 in human fetal PGCs.

A Immunofluorescence of primordial germ cells and somatic cells for detecting H3K4me1 (green), VASA (red), OCT4 (yellow) and DAPI (blue) proteins. Fetal ovaries and testis were stained. Scale bar 10 μm.

B Quantification of H3K4me1 signal in human germ and somatic cells. The error bars represent standard deviation.

Supplementary Figure 4. Chromatin changes of H3K9me3 in human fetal PGCs.

A Immunofluorescence of primordial germ cells and somatic cells for detecting H3K9me3 (green), VASA (red), OCT4 (yellow) and DAPI (blue) proteins. Fetal ovaries and testis were stained. Scale bar 10 μm.

B Quantification of H3K9me3 signal in human germ and somatic cells. The error bars represent standard deviation.

Supplementary Figure 5. The expression of Prmt5 in human fetal gonads. Immunofluorescence of primordial germ cells and somatic cells for detecting PRMT5 (green), VASA (red), OCT4 (yellow) and DAPI (blue) proteins. Scale bar 10 μm. (*: Heterogeneity in VASA staining).

A Fetal testis from 7 to 13 weeks of gestation

B Fetal ovaries from 7 to 13 weeks of gestation

C Quantification of nuclear and cytoplasmic PRMT5 staining in fetal testis and ovaries.

Supplementary Figure 6. The expression of DHX38 in human fetal gonads. Immunofluorescence of primordial germ cells and somatic cells for detecting DHX38 (green), VASA (red), OCT4 (yellow) and DAPI (blue) proteins. Scale bar 10 μm.

A Fetal ovaries from 6 to 13 weeks of gestation

B Fetal testis from 6 to 13 weeks of gestation.

Supplementary Figure 7. Quantification of the overall histone marks in human germ cells. The error bars represent standard deviation**.** Somatic cells normalized to 1.0.