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Original research

Genome wide abnormal DNA methylome of human blastocyst in assisted reproductive technology

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ABSTRACT

Proper reprogramming of parental DNA methylomes is essential for mammalian embryonic development. However, it is unknown whether abnormal methylome reprogramming occurs and is associated with the failure of embryonic development. Here we analyzed the DNA methylomes of 57 blastocysts and 29 trophectoderm samples with different morphological grades during assisted reproductive technology (ART) practices. Our data reveal that the global methylation levels of high-quality blastocysts are similar (0.30 ± 0.02, mean ± SD), while the methylation levels of low-quality blastocysts are divergent and away from those of high-quality blastocysts. The proportion of blastocysts with a methylation level falling within the range of 0.30 ± 0.02 in different grades correlates with the live birth rate for that grade. Moreover, abnormal methylated regions are associated with the failure of embryonic development. Furthermore, we can use the methylation data of cells biopsied from trophectoderm to predict the blastocyst methylation level as well as to detect the aneuploidy of the blastocysts. Our data indicate that global abnormal methylome reprogramming often occurs in human embryos, and suggest that DNA methylome is a potential biomarker in blastocyst selection in ART.

Introduction

Epigenetic information plays critical roles during animal development (Pujadas and Feinberg, 2012; Baubec et al., 2015; Lee et al., 2015; Schubeler, 2015). The plasticity of epigenome enables cell differentiation, organogenesis and animal development. On the other hand, proper epigenomic pattern at certain developmental stage is also required to ensure the totipotency of early embryos in animals (Baubec et al., 2015). Genome-wide DNA demethylation occurs during early embryogenesis in both human (Guo et al., 2014; Smith et al., 2014) and mouse (Smith et al., 2012; Wang et al., 2014). Manually disturbing the DNA methylome reprogramming by genetic knock-out of DNA methyltransferases (DNMTs) or Tet3 in mouse results in a failure of embryonic development (Li et al., 1992; Okano et al., 1999; Gu et al., 2011), indicating the importance of DNA methylome in mammalian development.

At present, approximately 12% of women of childbearing age in the United States have used assisted reproductive technology (ART) (National Collaborating Centre for Women's and Children's Health (UK), 2012; Centers for Disease Control and Prevention, 2014). During natural pregnancy, the fertilized human eggs often

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abnormally develop, resulting in the miscarriage (Evers, 2002; Macklon et al., 2002). Likewise, during ART practice, significant proportions of human early embryos defectively develop and fail in producing the birth alive (Heitmann et al., 2013; Hill et al., 2013). Genetic instability is speculated to be part of the reason, and indeed many kinds of mutations and chromosome errors are found in a proportion of these defective human embryos (Handyside et al., 1992; Wells and Delhanty, 2001). The frequency of aneuploidy in blastocysts significantly increased for women older than 35 (Franasiak et al., 2014a, b). To avoid the genetic defects in early human embryos, preimplantation genetic diagnosis (PGD) has been widely used through the removal of a few cells from an embryo by biopsy and subsequent genetic analysis (Handyside et al., 1992; Wells and Delhanty, 2001). PGD for patients with good prognosis have resulted in the increased implantation and delivery rates (Forman et al., 2013; Scott Jr. et al., 2013). Nevertheless, the majority of human embryos fail in producing birth alive for unknown reasons. Although DNA methylation plays important roles during embryogenesis, it is unknown whether abnormal methylome reprogramming occurs during human embryonic development.

Results

Global abnormality of DNA methylome in human blastocyst

Since DNA methylome reprogramming is highly associated with embryonic development in animals (Jiang et al., 2013; Wang et al., 2014), the precise reprogramming is, theoretically, important in determining embryonic condition. To investigate whether the abnormal methylome reprogramming occurs in human embryos, we analyzed the methylomes of blastocyst of various grades based on Gardner morphological blastocyst grading system including the ICM grade and the trophectoderm (TE) grade (Fig. 1A) (Hardarson et al., 2012). DNA methylomes at base resolution were examined by using a single blastocyst (Fig. S1A). We analyzed the methylomes of total 57 blastocysts with different morphological grades including high morphological grade (AA) blastocysts, middle grade (AB, BA, or BB) blastocysts and low grade (CC, BC or CB) blastocysts individually (Table S1). Two representative images of high grade (AA) and low grade (CC) blastocysts were presented in Fig. S1B. We calculated the average methylation levels of each blastocyst in different sequencing depth and found that the methylation levels were consistent in different depth (Table S2), which indicated that our method could capture the accurate methylation level with low sequencing depth. Our data reveal that the global methylation levels of 12 AA blastocysts are similar (0.30 ± 0.02), ranging from 0.27 to 0.32 (Fig. 1B and Table S1). Surprisingly, the methylation levels of 12 (among 20) middle quality blastocysts are variant and do not fall within the range of 0.30 ± 0.02 (Fig. 1B and Table S1). Furthermore, the methylation levels of 22 low quality blastocysts (among 25) differ from AA blastocysts (Fig. 1B). The methylation levels of low-quality blastocysts are divergent, ranging from 0.23 to 0.46. Compared to the high-quality blastocysts, the low-quality blastocysts are more inconsistent in terms of methylation level (Fig. 1B). We further applied principal component analysis (PCA) to examine the variations of DNA methylation pattern among different blastocysts. The data show that the AA blastocysts are closely clustered together (Fig. 1C, blue circle), indicating that the variations among AA blastocysts are small (Fig. 1C). For middle grade blastocysts, most of the blastocysts with methylation level falling within the range of 0.30 ± 0.02 are similar to AA blastocysts (Fig. 1C, within blue circle). As for the low-quality blastocysts, the majority of them distribute outside of the blue circle, and the variations among them are much larger (Fig. 1C). The data

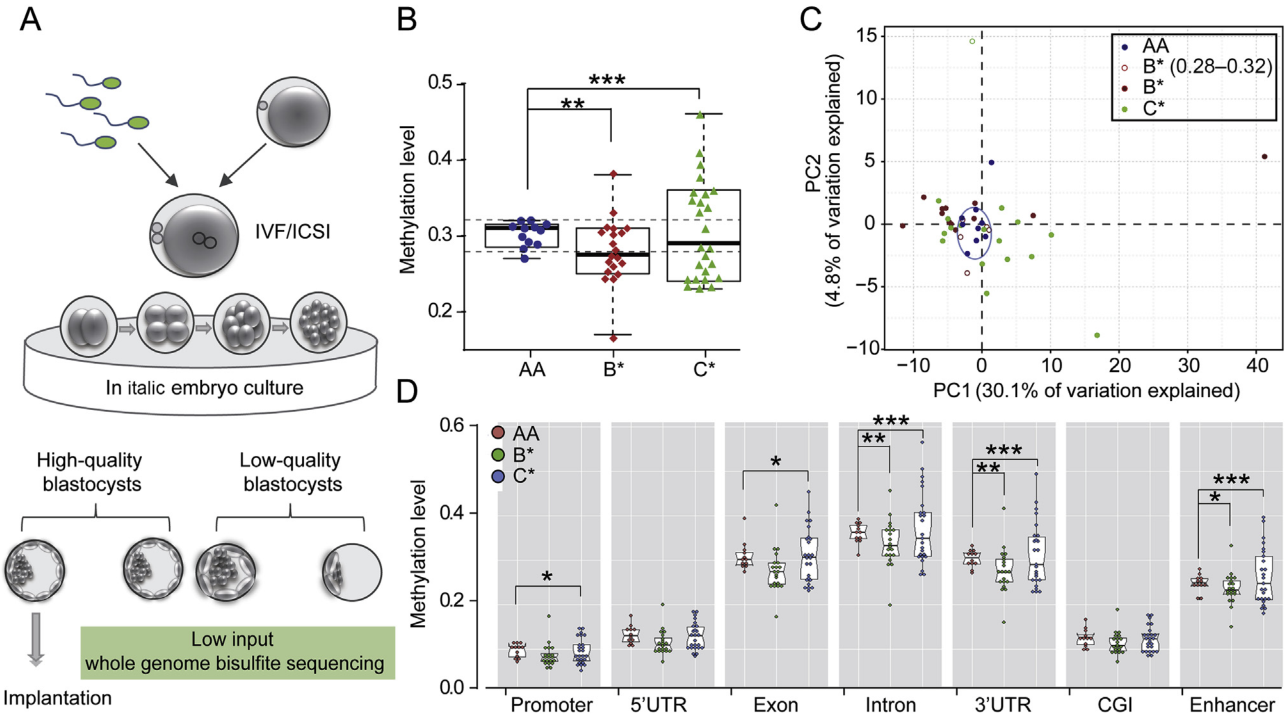

Fig. 1. Global methylome instability in human early embryos. **A**: The schematic diagram of the study. **B**: The methylation level of high-quality (AA), middle-quality (B*) and low-quality (C*) blastocysts. 1-4AA and 1-2CB blastocysts were derived from the same parents, as were the 1-6AA and 2-2CC blastocysts. The *P* value of the homogeneity of variance between AA and B* is 0.004 (**), and the *P* value between AA and C* is 7×10^{-5} (***). **C**: PCA analysis according to the CpG methylome status of each blastocyst. PC1 represents 30.1% of variation and PC2 explains 4.8% of variation. **D**: Average methylation levels of various functional elements in different blastocysts. The *P* values of the homogeneity of variance between AA and B* or C*: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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demonstrate that the divergence of DNA methylome of blastocysts correlates with the morphological grades.

Additionally, the methylation pattern of functional elements in high-quality blastocysts is similar, while the pattern in each blastocyst of low-quality blastocysts is different (Figs. 1D, S1C and S1D; Table S3). Taken together, the data show that genome-wide abnormality of DNA methylome frequently occurs during human embryonic development.

Abnormal methylated regions are associated with development pathways

To gain a close-up view of the differences observed above, we investigated the differentially methylated regions (DMRs) between high-quality and low-quality blastocysts (Table S4). The average length of DMRs is around 2 kb (Fig. S2A). A significant proportion of DMRs locate in CpG islands (CGIs) or CGI shores (Fig. S2B and C). Functional enrichment analyses demonstrate that the genes with promoters locating in DMRs are enriched in many fundamental pathways critical for embryonic development, including cell cycle, DNA metabolism, chromosome localization and DNA modification (Fig. 2A). Genes associated with intergenic DMRs are enriched in specific developmental categories, such as neuron development and spinal cord patterning (Fig. 2A).

Interestingly, our data show that DNA methylation in enhancer regions are often falsely reprogramed in low-quality embryos. Gene ontology (GO) enrichment analysis for differentially methylated enhancers (Fig. S2D) shows the enriched categories in developmental and metabolism pathways (Fig. S2E). Fig. 2B shows that the enhancer for *IDH2* gene, encoding a metabolism enzyme which can regulate the oxidization of 5mC (Kaelin and McKnight, 2013), is hyper-methylated in CC blastocysts (Fig. 2B). Similarly, the expression of *IDH2* in three CC blastocysts is much lower than that in three AA blastocysts (Fig. 2C). For another example, cell cycle gene *CDK10* also has differentially methylated enhancers, and presents differential expression between high- and low-quality blastocysts (Fig. S2F and G). Furthermore, the global transcriptomes of CC blastocysts are also different from AA blastocysts (Fig. S2H). Taken together, our results suggest that abnormal methylome may affect the developmental potential of early embryos.

Methylome status is associated with the live birth rate in ART treatment

In ART treatments, AA blastocysts are ideal for embryonic transfer, which produce 39% live birth rate in Peking University Third Hospital. However, only a minority (33%) of the fresh elective single embryonic transfers (eSet) are AA blastocysts in our hospital, while the majority of the blastocysts for embryonic transfer (52%) are middle-quality B* blastocysts (AB, BA and BB) (Fig. S3A). The live birth rate for either AB/BA blastocysts or BB blastocysts is approximately up to 28% (Fig. S3B). A small proportion of patients used the low-quality C* blastocysts (BC and CB) for embryonic transfer, which resulted in nearly 4% live birth rate (Fig. S3B). Previously, the tested CC grade blastocysts were unable to produce any live birth (Heitmann et al., 2013; Hill et al., 2013), and thus they were not used for embryonic transfer in our hospital. Considering that AA blastocysts have the highest live birth rate and uniform methylome, we regarded the methylome of AA blastocysts as good epigenomic status and used the average methylation level of AA blastocysts (0.30 ± 0.02) as the control. Interestingly, the proportion of blastocysts with a methylation level falling within the range of 0.30 ± 0.02 in different grades is correlated with the live birth rate for that grade (Fig. 3A, Pearson Correlation Coefficient, $r = 0.93$). The data suggest that DNA methylome status is associated with the live birth rate in ART.

DNA methylome examination of cells biopsied from blastocyst

PGD has been used for more than 20 years to screen genetic diseases during ART practice. The removal of cells by biopsy from the trophectoderm has been used for the diagnosis of genetic mutations or chromosomal errors before embryonic transfer to the uterus (Handyside et al., 1992; Wells and Delhanty, 2001). Therefore, we used the cells removed by biopsy from TE in blastocyst to analyze the DNA methylome. The methylomes of 29 biopsied TE samples were profiled, 26 of which have paired methylome data of the blastocysts (Table S1). Our data show that the methylation levels of TE from high-quality and middle-quality blastocysts are similar to the paired blastocysts (Fig. S3C). The methylation levels of the TEs from low-quality blastocysts are usually comparable to the levels of the paired blastocysts, except that the levels of the TEs in a few samples are higher than those in the paired blastocysts, but the levels are still significantly different from the control level (0.30 ± 0.02) (Fig. S3D and Table S1). Thus, our results show that the DNA methylomes of a few cells from TE in high and middle grades can indicate the methylome of entire blastocyst. Notably, the *P* value of variation between high-grade and low-grade TEs is 0.03, and the *P* value between high grade and middle grade is 0.05. The data indicate that the methylation status of TE is also correlated with the morphological grade of blastocysts (Fig. 3B). Therefore, as for the high- and middle-quality blastocysts, we can use the methylomes of a few biopsied cells from TE to predict the DNA methylome of the blastocyst before the embryonic transfer. Since low-quality blastocysts will not be used in ART practices, it is unnecessary to examine its methylomes.

Aneuploidy analysis from methylome data

Aneuploidy is frequently observed in human embryos. PGD has been widely used through the removal of a few cells from an embryo by biopsy and subsequent genetic analysis (Handyside et al., 1992; Wells and Delhanty, 2001). PGD has resulted in the increased implantation and delivery rates (Forman et al., 2013; Scott et al., 2013; Franasiak et al., 2014a, b). PGD for aneuploidy screening (PGD-AS) has been applied in ART practices by array-based comparative genomic hybridization (arrayCGH) and single-nucleotide polymorphism (SNP) array approaches (Dahdouh et al., 2015). More recently, next-generation sequencing has been introduced into *in vitro* fertilization (IVF) field (Fiorentino et al., 2014). DNA methylation data have also been used to evaluate the copy number of chromosomes (Oda et al., 2009; Feber et al., 2014). Therefore, we aimed to use our DNA methylome data to analyze the chromosome copy number variations (CNVs) in blastocysts and the biopsied TE cells. Notably, aneuploidy was present in 22 blastocysts (Fig. 3C; Table 1), about 30% of all examined samples, which is consistent with the ratio discovered by traditional PGD (Fragouli et al., 2011). Our data show that some blastocysts with normal methylation level still present with aneuploidy (Fig. 3D), indicating that aneuploidy does not affect DNA methylation. Notably, the results in the paired blastocyst and biopsied TE samples are highly consistent (Table 1). Therefore, we can use DNA methylome from biopsied samples to predict the DNA methylome pattern and chromosome pattern, which may be useful for TE selection in the future.

Discussion

In this study, we performed whole genome bisulfite sequencing

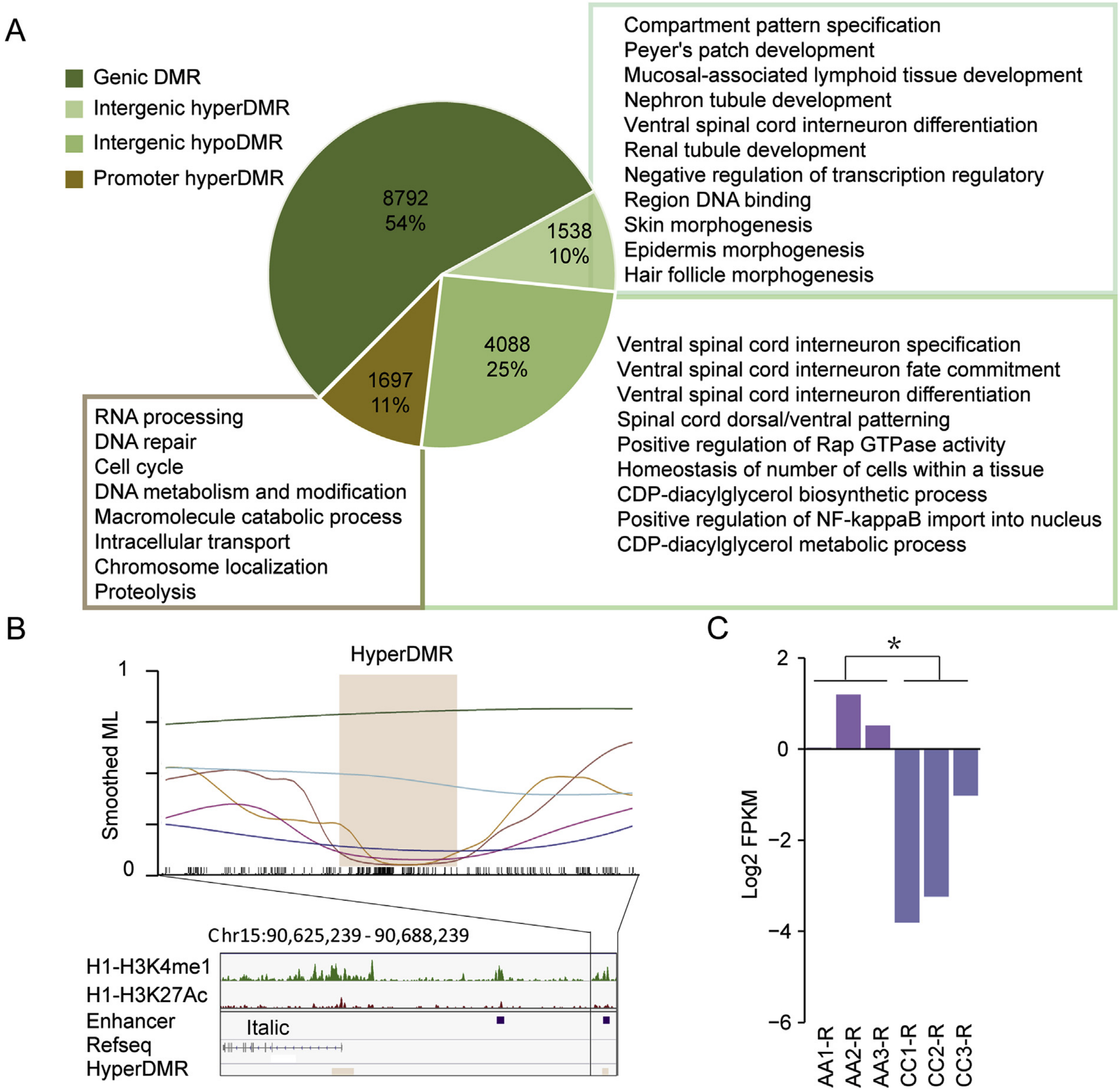

Fig. 2. Functional analysis of altered methylation reprogramming. **A**: Functional enrichment of differentially methylated regions between high-quality and low-quality blastocysts. Genes with promoters located in DMRs were used for DAVID GO enrichment analysis. The *cis*-regulatory functions of intergenic DMRs were analyzed with GREAT tools. GO biological pathways with $P < 0.05$ were considered as the significant difference. **B**: The enhancer near *IDH2* is differentially methylated. The smoothed methylation levels of high-quality and low-quality blastocysts are showed with extension ±5 kb. The smoothed ML analysis was according to the previous method (Hansen et al., 2011). Red, orange and purple lines indicate 1-4AA, 2-4AA and 1-6AA, respectively. Blue, cyan and blue violet lines indicate 9-3CC, 2-2CC and 8-3CC, respectively. The DMR is indicated with pink shading. Short black bars indicate the location of CpG sites. **C**: The relative mRNA expression level of *IDH2* in AA blastocysts and CC blastocysts. Three RNA-seq replications were performed for each group. *, $P = 0.03$.

for 57 human preimplanted blastocysts as well as 29 biopsied TE samples individually. We found that abnormal methylome reprogramming often occurs in human embryos (Fig. 1). We showed that DNA methylome examination in biopsied TE can predict epigenetic status and chromosome aneuploidy for the entire blastocyst (Fig. 3).

The global methylome abnormality frequently takes place in human blastocyst, and is associated with low live birth rate. Previous studies in human diseases including cancer show that the global methylation levels in pathological tissues have limited (or no) change compared to the normal tissues (Baylin and Jones, 2011; Hansen et al., 2011; Shen and Laird, 2013). Considering that genetic manipulation of *DNMTs* in mouse can lead to the failure of embryonic development (Li et al., 1992; Okano et al., 1999), the global abnormal methylome may be an important factor, which can result in the failure of human embryonic development. Indeed, falsely reprogrammed methylation regions are highly enriched in development related pathways (Fig. 2), suggesting that the abnormal methylome may affect the developmental potential of embryos.

Right now, we have not revealed the mechanism behind the abnormal methylome. Various pathways in different embryos may involve the causes of improper reprogramming. We have shown that the aneuploidy is not associated with DNA methylation in blastocyst (Fig. 3D). The advanced maternal age is associated with the rate of aneuploidy in blastocysts (Franasiak et al., 2014a, b). Probably, epigenetic status in blastocysts may also be affected by the maternal age. There is the likelihood that an improper microenvironment ultimately leads to an altered methylome in blastocysts since the environment has the ability to influence epigenetic

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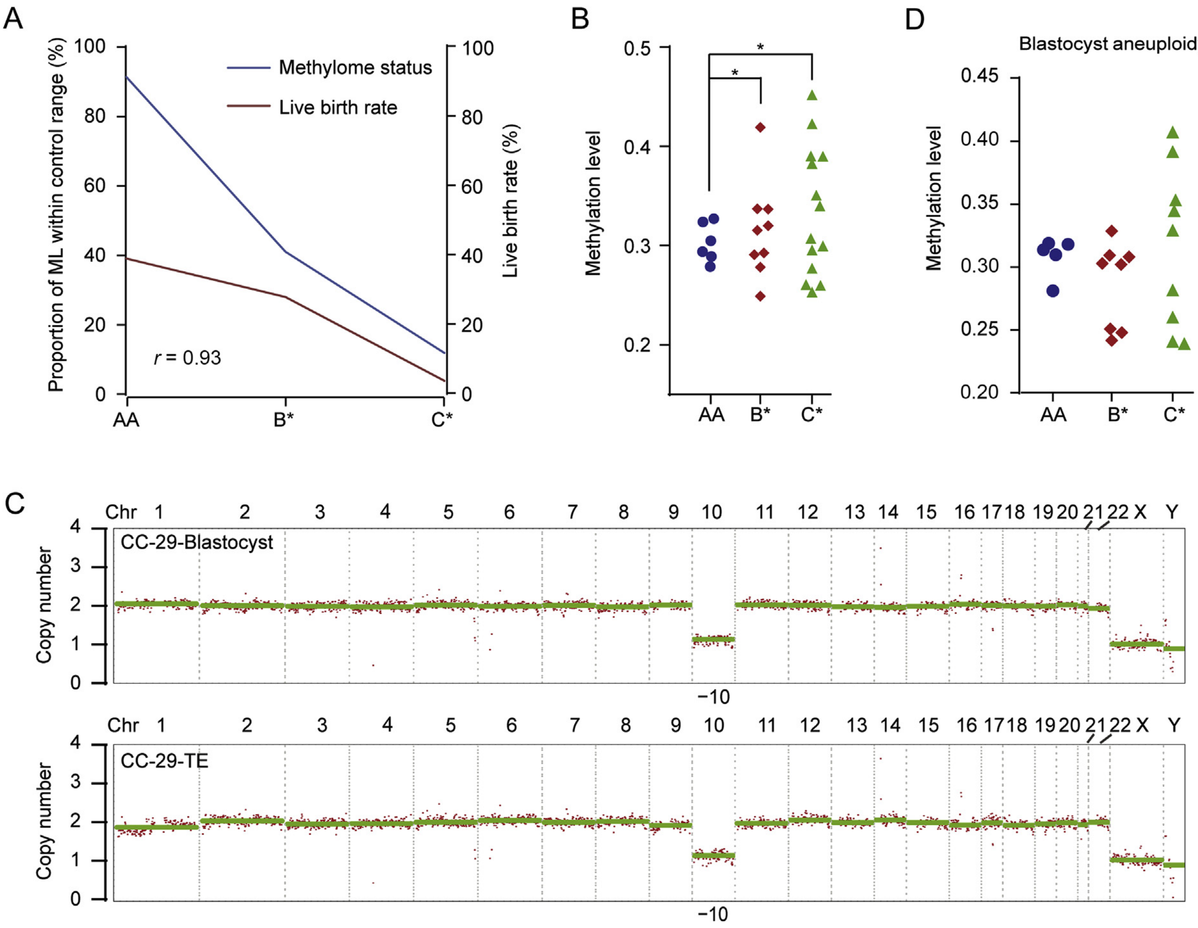

Fig. 3. DNA methylome examination in human early embryos. **A**: The blue line indicates the proportion of blastocysts with methylation levels falling within 0.30 ± 0.02. The red line indicates the live birth rate of the blastocysts with different grade. The result was from the patients using fresh embryos. The live birth rate of BC and CB is used to replace the live birth rate of BC, CB and CC blastocysts. The live birth rate of BC and CB should be higher than that of BC, CB and CC altogether. ML, methylation level. **B**: The methylation level of TE biopsied from high-quality (AA), middle-quality (B*) and low-quality (C*) blastocysts. *, $P < 0.05$. **C**: The graphical illustration of chromosome copy number was detected from our DNA methylome data. **D**: The methylation level of blastocysts with aneuploidy.

Table 1
Aneuploidy results for the embryos by using DNA methylome data. B* means middle-quality (AB, BA or BB), C* means low-quality (CC, BC or CB).

Embryo quality	Sample	Aneuploid	Paired sample	Aneuploid
AA	2-4AA	−19	6-5AA	−1p
	25-3AA	+5q, −9q	6-5AA-TE	−1p
			17-5AA	−22
			17-5AA-TE	−22
			19-4AA	−16
			19-4AA-TE	−16
B*	14-4BB	−13	11-5BB	−22
	54-5BB	−14	11-5BB-TE	−22
	32-6BA	+22	15-4BA	−20, −19
	13-5BB	−15, +17	15-4BA-TE	−20, −19
	31-5BB	−21, −22		
	18-5BB	−14		
C*	9-3CC	+7, X	29-3CC	−10
	2-2CC	−21	29-3CC-TE	−10
	36-3CC	+6	30-3CC	−12
	41-3CC	+16	30-3CC-TE	−12
			35-4CC	+16
			35-4CC-TE	+16
			50-4CC	XXY
			50-4CC-TE	XXY
			51-4CC	+4
			51-4CC-TE	+4

states (Jirtle and Skinner, 2007). Frozen-thawed embryo transfer cycles may affect the stability of some proteins in early embryos, which could also have the impact on the DNA methylome patterns. It is our hope that the optimization of embryo culture conditions during *in vitro* fertilization will facilitate the methylation reprogramming and will improve the efficiency of ART treatment soon. In the future, more studies should be done to investigate the cause of the abnormality of DNA methylomes in human early embryos.

Right now, the outcome at birth is defined as a successful pregnancy. However, the consequences of ART practice for the baby's later life remains to be considered. Precious work has suggested that the intrauterine environment is associated with epigenetic programming of the fetal metabolism and predisposition to chronic metabolic disorders later in life (Lehnen et al., 2013). Epimutation, especially these in imprinting disorders, is wildly observed after ART (Tee et al., 2013). Therefore, a full understanding of the cellular and molecular biology of human reproduction must include a study of epigenetics and genomic imprinting. Furthermore, epigenetic variation during ART practice should be taken into consideration before embryo transferring.

PGD has been widely used to detect genetic mutations or chromosome errors before embryonic transfer to the uterus during ART clinic practices (Handyside et al., 1992; Wells and Delhanty, 2001). We show that DNA methylome examination can also detect aneuploidy in blastocysts. Our data further demonstrate that

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embryos with better methylome status are associated with a higher live birth rate (Fig. 3A). Furthermore, our data suggest that epigenomic examination in blastocyst can determine the epigenomic status and euploid chromosome of the blastocyst prior to the embryonic transfer. Therefore, the methylome examination in blastocyst may have advantage compared to the traditional PGD method, and improve the efficiency of ART. Currently, 12% of women use ART services in the United States. In the future, proper clinic trial is needed to evaluate the value of DNA methylome examination method. We wish that the methylome examination in blastocyst might improve the efficiency of ART practices.

Material and methods

Human samples

The human tissue collection and study procedure in this study were approved by the Institutional Review Board at Peking University Third Hospital (Research license 2012SZ015). The methods closely followed the guidelines legislated and posted by the Ministry of Health of the People's Republic of China. The patients were informed of all details of the procedure, including sample utility and research destination. Patients voluntarily signed an informed consent document. Human embryos at the blastocyst stages were donated by the couples who had conceived at least one healthy baby by ART treatment. These donor couples, whose infertility is purely due to female tubal factors, had a healthy baby through the IVF cycle already. They then donated the surplus frozen embryos for research with the written informed consents signed by them. Embryos were then graded according to Gardner morphological blastocyst grading system before collected for further methylation study.

The human embryos prepared for blastocyst biopsy were treated firstly at early blastocyst stage. One hole was made in the zona pellucida of these embryos opposite to inner cell mass (ICM) using laser pulse (ZILOS-tk, Hamilton Thorne Biosciences, USA). On day 6, the fully developed blastocysts were grouped and transferred into biopsy buffer medium (G-MOPES PLUS, Vitrolife, Sweden). The ICM was fixed at the 9 o'clock position by the holding pipette, and the hached TE cells were stretched from the hole by a biopsy pipette with a 25 μm diameter. A few cells were removed from the blastocysts using laser pulse. The biopsied TE cells and the surplus blastocyst were then processed to methylome analysis.

Whole genome bisulfite sequencing

Rather than mapping the methylome by pooling multiple blastocysts together, we investigated the methylome of each blastocyst individually. DNA methylation libraries of human single blastocyst were constructed with our modified library generation method termed as “One Tube” method. Briefly, single blastocyst was lysed and then fragmented by sonication. The fragmented DNA was end-repaired, dA-tailed and ligated to cytosine methylated Illumina Truseq adapter. Bisulfite conversion reaction was performed directly on the ligation mix with 0.5% unmethylated λ DNA spiked-in. PCR amplified library was purified and sequenced on Hiseq2000 or Hiseq3000 platform (Illumina, USA).

mRNA sequencing

Blastocyst RNA was amplified and reverse-transcripted with REPLI-g WTA Single Cell Kit (Qiagen, USA). RNA-seq libraries were constructed with NEBNext® Ultra™ RNA Library Prep Kit for illumine (NEB, USA) according to manufacture instruction. QC-passed libraries were then sequenced on Hiseq2500 platform with pair-end module. Methyl-seq and mRNA-seq data have been deposited in the Genome Sequence Archive (GSA) under project number PRJCA000248.

Sequencing data processing

Sequencing reads were trimmed to remove the low-quality reads and reads containing adapters. Sequencing reads were trimmed with default setting of Trimmomatic software (Bolger et al., 2014) to remove the adapters and low-quality reads. Trimmed reads were aligned to human reference hg19 by using Bismark (v12.5) (Krueger and Andrews, 2011). PCR duplications were removed with Picard (http://broadinstitute.github.io/picard/) and the overlapped regions in uniquely mapped paired reads were clipped with clipOverlap function of BamUtil (http://genome.sph.umich.edu/wiki/BamUtil: clipOverlap). CpG and non-CpG methylation levels were extracted with mpileup function of Samtools (v0.1.19) (Li et al., 2009). Strands were merged to calculate the CpG methylation level per site. Average methylation level in each stage was the mean of methylation levels of each site. PCA is often used to emphasize grouping structure in the data. We performed PCA analysis on the samples' methylation profiles by R package methylKit (Akalin et al., 2012). For PCA analysis, the methylomes with the genomic coverage higher than 15% were included. For DMR analyses in Figs. 2 and S2, we used the R package bsseq, which is a smoothing local likelihood method that shows precise results even with low coverage data as well as have the ability to handle biological replicates (Hansen et al., 2012). DMRs contain at least 5 CpGs and the difference level between two groups higher than 0.2 were used for further analyses.

For mRNA-seq data, adapter-containing and low-quality reads were trimmed and then aligned with TopHat (Trapnell et al., 2009). The unique reads were used to calculate the Fragments Per Kilobase of transcript per million mapped reads (FPKM) with Cufflinks v2.0.2 (http://cufflinks.cbcb.umd.edu). Differential gene expression between high- and low-quality blastocysts was calculated with DESeq using the default parameters (Anders and Huber, 2010).

The chromosome number was deducted by R package HMMcopy (Ha et al., 2012), which make copy number estimation based on read depth of the whole genome data in fixed interval with additional GC and mappability correction. The copy numbers were then segmented and classified with a robust Hidden Markov Model. Here we divided the genome into non-overlapping windows of 1 Mb, and assigned the median autosomal read count corresponding to copy number 2. Chromosomal gain (copy number > 2) and loss (copy number < 2) were seen as horizontal green bars above and below, respectively, the copy number state of 2. Embryos were diagnosed as normal or euploid if the generated plot showed no gain or loss.

Bartlett's test

Bartlett's test is used to test if k samples are from populations with equal variances (Snedecor and Cochran, 1989). Equal variance across populations is called homoscedasticity. In our work, we focused on the analysis of variance in different group of blastocysts, assuming that variances are equal across groups. The Bartlett's test can be used to verify that assumption.

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Supplementary data

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