Methylation Status of H19/IGF2 Differentially Methylated Region in in vitro Human Blastocysts Donated by Healthy Couples

Marzieh Derakhshan-Horeh¹, Farid Abolhassani*¹,¹¹, Farnoosh Jafarpour², Ashraf Moini³,⁴, Khadijah Karbalaie⁵, Sayed Morteza Hosseini², Somayeh Ostadhosseini² and Mohammad Hossein Nasr-Esfahani*²,⁵,⁶

¹Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran; ²Department of Reproductive Biotechnology, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, Academic Center for Education, Culture and Research (ACECR), Isfahan, Iran; ³Department of Endocrinology and Female Infertility, Reproductive Biomedicine Research Centre, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran; ⁴Department of Obstetrics and Gynecology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran; ⁵Department of Cellular Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran; ⁶Isfahan Fertility and Infertility Center, Isfahan, Iran

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ABSTRACT

Background: Imprinted genes are a unique subset of few genes, which have been differentially methylated region (DMR) in a parental origin-dependent manner during gametogenesis, and these genes are highly protected during pre-implantation epigenetic reprogramming. Several studies have shown that the particular vulnerability of imprinting genes during suboptimal pre- and peri-conception micro-environments often is occurred by assisted reproduction techniques (ART). This study investigated the methylation status of H19/IGF2 DMR at high-quality expanding/expanded human blastocysts donated by healthy individuals to evaluate the risks linked to ART.

Method: Methylation levels of H19/IGF2 DMR were analyzed by bisulfite conversion and sequencing at 18 CpG sites (CpGs) located in this region. Result: The overall percentage of methylated CpGs and the proportion of hyper-methylated clones of H19/IGF2 DMR in analyzed blastocysts were 37.85±4.87% and 43.75±5.1%, respectively. For validation of our technique, the corresponding methylation levels of peripheral human lymphocytes were defined (49.52±1.86% and 50%, respectively). Conclusion: Considering the absence of in vivo-produced human embryos, it is not possible to conclude that the methylation found in H19/IGF2 DMR is actually normal or abnormal. Regarding the possible risks associated with ART, the procedures should be optimized in order to at least reduce some of the epigenetic risks. DOI: 10.6091/21.1.16

Keywords: Blastocyst, Genomic imprinting, H19/IGF2 DMR, Human, Reproductive technique

INTRODUCTION

Methylation of cytosine in the 5' position in CpG dinucleotides, i.e. DNA methylation, is a crucial epigenetic control mechanism in mammals. The most dramatic changes in DNA methylation occur during gametogenesis and early embryo development. For example, the overall DNA methylation of CpG islands in mouse sperm is in the range of 80-90%, which is higher than any other cell in this organism. The maternal genome, however, contains much lower level of DNA methylation (~40%). Accordingly, paternal genome is actively and rapidly demethylated through the oxidation of 5-
methylenic to 5-hydroxymethylcytosine by ten-eleven translocation family of enzyme, while the maternal genome is demethylated in a replication-dependent manner[1].

In spite of the global DNA demethylation following fertilization, there is a unique subset of genes, approximately 150 genes in human and mouse[2], which are specifically protected from DNA demethylation[3,5]. This class of genes is associated with the genomic differentially methylated regions (DMRs) in a parental origin-dependent manner during gametogenesis. It has been well established that the parent-of-origin specific DNA methylation, known as genomic imprinting, is crucially important for normal embryonic development. Therefore, any interference with imprint acquisition and/or maintenance will result in ill or fatal phenotypes of the resultant embryos[6].

In human, most of the imprinted genes are arranged in clusters[7]. As an example, several clusters are located on chromosome 11p15.5 containing two important imprinted genes, IGF2 and H19. The DMR of H19/IGF2 constitutes an imprint control region (ICR) that regulates nearby imprinted genes. ICR1/H19 DMR is located between IGF2 and H19 in order to regulate the IGF2 and H19 expression in a reciprocal manner. ICR1 prevents the activation of IGF2 promoters through interaction with the zinc finger protein CCCTC-binding factor (CTCF)[8,10]. Therefore, when paternally inherited, DNA methylation inhibits CTCF binding, and ICR1 activity is blocked; hence, IGF2 is expressed[11]. However, when maternally inherited, ICR1 remains methylation-free, and H19 is expressed (Fig. 1).

Russell-Silver syndrome (RSS) is a syndrome with pre- and post-natal growth retardation, and Beckwith–Wiedemann syndrome (BWS) is characterized by prenatal and postnatal overgrowth[12]. Hypomethylation of ICR1 is observed in RSS patients[13,14], while hypermethylation of ICR1, which is associated with increased IGF2 expression, is observed in BWS patients[15]. It has been shown that paternally expressed genes (such as IGF2) tend to increase fetal growth, whereas those expressed maternally, such as H19, restrict fetal growth[16-18].

Although assisted reproductive techniques (ARTs) are now well-established and globally applied, several epidemiological studies have shown the association of ART with the increased incidence rate of certain imprinting disorders such as BWS and RSS[19]. This phenomenon has been mainly attributed to the coincidence between gamete and embryo in vitro manipulations events and the normal pattern of epigenetic reprogramming, which begins at fertilization and continues during pre-implantation embryo development[6,20,21].

Despite several investigations in the gametic DMRs (gDMRs) in human pre-implantation embryos, there is still space for further studies. The reason is that in most studies, embryos used were derived from leftover of ART cases and may do not have the best quality or may have been derived from infertile individuals. Considering the possible consequences of reprogramming errors, it is essential to study the

![Diagram](image)

**Fig. 1.** Diagram of H19/IGF2 imprinted domain regulated by ICR1. A) Diagram of H19/IGF2 imprinted domain regulated by ICR1 (green rectangles), down-stream enhancers (red triangles), and CTCF (yellow hexagons). Differential methylation of ICR mediates with differential binding of CTCF results in the differential expression of H19 and IGF2; B) ICR1 is formed of tandem repeat elements (B-type repeats, orange boxes) containing CTCF-binding motifs; C) Part of sequence context of B1 repeat assessed in our study [18 CpG sites in a 220-bp fragment of ICR1 (AF125183; 7877–8096)] on paternal (methylated) and maternal (unmethylated) allele in lymphocyte. Unmethylated cytosine in CpGs on maternal allele is converted to thymine (violet color). Methylated cytosine in CpGs on maternal allele remains unchanged (orange CpGs).
normal gDMR DNA methylation status of human blastocyst derived from healthy couples. Therefore, in this study, we determined the methylation status of ICR1 in high-quality blastocysts donated by couples who desire family balancing with proven fertility.

MATERIALS AND METHODS

Informed consents and ethical approval
This study was approved by the Ethics Committee of Tehran University of Medical Sciences (no. 92-03-30-24088, 2014/6/7) and Ethical Committee of Royan Institute (Tehran, Iran). All embryos were collected from patients referring to the Isfahan Infertility Center (Isfahan, Iran), and a written informed consent was obtained.

Source of human blastocyst
Human blastocysts were donated from patients with supernumerary blastocysts referring to the Isfahan Infertility Center for family balancing. A total of 20 expanded or expanding blastocysts were obtained from couples with at least two children of the same sex. Antagonist protocol (Cetroide, Serono) in combination with SinalF (SinaClon, Tehran, Iran) and Menogon (Ferring, Germany) was used for ovulation induction. Ovulation was induced with 10,000 IU of human chronic gonadotropin (IBSA, Switzerland) when three dominant follicles greater than 17 mm were observed in vaginal ultrasound scan. Intra cytoplasmic sperm injection and pre-implantation genetic diagnosis were carried out based on the standard protocols, and G5 series sequential media (Vitrolife, Gothenburg, Sweden) was used for all the procedures. Blastocysts were scored according to Gardner et al. grading system. After transferring the fresh embryos and cryopreservation of the embryos, the remaining blastocysts were used for this study after receiving a signed informed consent forms from the patients. The embryos of the patients who were not agreed to donation were discarded. The pre-implantation genetic diagnosis results were not revealed to the research group because: i) embryos were scarce, ii) in general ART practices, information on chromosomal status of embryos is not available, and iii) mosaicism is a typical feature of ART embryos. For epigenetic analysis, hatched blastocysts were used. Hatching was induced with the aid of a pipette or removal of zona pellucida with the aid of Tyrode’s acid. Zona-free blastocysts from each couple were pooled and stored in 0.2-ml microtubes at -80°C. Care was taken to make sure there is no granulosa contamination.

DNA methylation analysis
The DNA methylation of H19/IGF2 DMR was determined by bisulfite conversion and sequencing as described by Borghol et al. on a total of 20 blastocysts donated from 6 couple and one million peripheral blood lymphocytes derived from 5 volunteers. Lymphocyte was used as control to obtain the correct pattern of imprinted gene methylation. The genomic DNA from lymphocytes was extracted by salting-out method. The pools of small number of blastocysts (2-3/pool) from the same patient were used per replicate. Blastocysts were thawed and directly placed in a lysis solution (50 mM EDTA, 50 mM Tris, pH 8, 3 µg of RNA carrier, and 0.14 µg/µl proteinase K) in a final volume of 40 µl and then incubated at 55°C for 2 h. Complete bisulfite conversion and DNA clean-up were performed by the EpiTect® Bisulfite Kit (Qiagen, Germany) based on the manufacturer’s. After treatment with bisulfite and purification, DNA was immediately used for nested PCR.

We examined 18 CpG sites in a 220-bp fragment of human blastocyst DNA methylation binding site. Primers specific for bisulfite-converted DNA were as follows: external forward: 5’-GAGTTYGGGGTTTTTGTATAGTAT-3’; external reverse: 5’-CTTAAATCCAAACCATAACACTA-3’; internal forward: 5’-TATATGGATTTTTGGAGGT TTTT-3’; internal reverse: 5’-ATAATATCCTATTCCCAAAATACCC-3’.

The PCR master mix was prepared according to Herman et al. with some modifications. Briefly, 30 pmol (300 ng) of each forward and reverse primer was used for PCR reactions with 1x ammonium sulfate buffer (CinnaGen, Iran, CG8108C), 6 mM MgCl2 (CinnaGen, TP7506C), 1.25 mM dNTP (CinnaGen, DN7603C), 0.6 U SmaTag (CinnaGen, TA8108C) and 10 mM 2-mercaptoethanol (Sigma, M7522, USA) in a 50-µl reaction volume. PCR reaction was carried out in an Eppendorf gradient thermal cycler with the following program: 95°C for 10 min, followed by 39 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min with a final extension at 72°C for 10 min. The first-round PCR product (2 µl) was used as DNA input for amplification in the second-round nested PCR mix with the following conditions: 95°C for 10 min, 39 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 20 min. Four independent nested PCRs were performed per template to avoid amplification bias, which may lead to preferential amplification of maternal or paternal DMRs. PCR products were sub-cloned into a pTZ57R/T cloning vector (InsTAcione™ PCR Cloning Kit, Fermentas, Lithuania) according to the...
The methylation status of the H19 DMR was determined by cloning and sequencing of bisulfite-treated DNA. To reduce amplification bias of maternally- or paternally-derived genome copies, the pools of a small number of blastocysts (2-3/pool) from the same patient were analyzed. In addition, more than 15 clones were sequenced per replicate for four independent PCR products. The efficiency of bisulfite PCR amplification from blastocysts was 66.6%.

**Statistical analysis**

The proportions of hyper-methylated clones and the percentages of overall methylation were determined to be normally distributed, with the homogeneity of variances (Leven’s test). To assure the accuracy of the results, the experiment was repeated at least four times.

**RESULTS**

**Methylation status of the H19/IGF2 DMR**

Our bisulfite sequencing protocol was validated on genomic DNA extracted from human peripheral blood after several independent experiments, indicating no bias among methylated or unmethylated alleles. Therefore, the methylation status of lymphocyte was used as a template for validation of methylation status of the embryos (Fig. 2A)\(^{24,27}\).

| CpG site | CpG position | Methylation (%) |
|----------|--------------|-----------------|
| 1        | 5            | 37.50           |
| 2        | 14           | 37.50           |
| 3        | 27           | 43.70           |
| 4        | 15           | 25.00           |
| 5        | 61           | 43.70           |
| 6        | 63           | 43.70           |
| 7        | 65           | 37.50           |
| 8        | 68           | 50.00           |
| 9        | 96           | 43.70           |
| 10       | 102          | 43.70           |
| 11       | 118          | 43.70           |
| 12       | 122          | 43.70           |
| 13       | 130          | 43.70           |
| 14       | 135          | 31.25           |
| 15       | 151          | 31.25           |
| 16       | 153          | 31.25           |
| 17       | 156          | 12.50           |
| 18       | 167          | 31.25           |

Fig. 2. Bisulfite sequencing analysis of H19/IGF2 DMR. The sequencing result of H19 DMR in a representative replicate from the A) lymphocyte and B) the blastocyst. Each row indicates a unique DNA clone. Filled and open square represent methylated and unmethylated CpGs, respectively. The number of clones is presented as percentage (%). In these replicates, the total number of clones is 16.
Table 2. DNA methylation summary overall sequences in a representative replicate located in ICR1 sequence in the blastocyst

| DNA methylation summary | The number of CpG overall sequences (180 cases of 288) (%) |
|-------------------------|----------------------------------------------------------|
| Unmethylated CpGs       | 62.5                                                     |
| Methylated CpGs         | 37.5                                                     |

Percentages of overall methylated CpGs at H19/IGF2 DMR

To identify DNA methylation patterns of H19/IGF2 DMR, the region including 18 CpGs was analyzed. The percentage of overall methylated CpGs of the blastocysts in the region was 37.85±4.87%. The detail of methylation status in ICR1 sequence in a representative replicate is presented in Figure 2B, Tables 1 and 2. Furthermore, the methylation pattern of lymphocyte was 49.52±1.86% (Fig. 2A).

Percentages of hyper-methylated clones of H19/IGF2 DMR

The clones with >50% of the CpGs methylated are considered as hyper-methylated, and strands lacked nine or more methylated CpGs were considered to be hypo-methylated[28,30]. Using these gauges, the percentage of hyper-methylated clones of H19/IGF2 DMR in the blastocysts was 43.75±5.1%, while this value for the lymphocyte was 50% (Fig. 2, Table 3).

DISCUSSION

Thirty six years after the birth of first child through in vitro fertilization and with the advent of new techniques, including cryo-preservation, intra cytoplasmic sperm injection, pre-implantation genetic diagnosis, and assisted oocyte activation, ART is now accounting for a considerable proportion of child birth. In some European countries, this proportion reaches more than 3.0%. However, epidemiological reports raise major concern and issues regarding the epigenetic consequences of ART manifested at different stages of pre-, peri- and post-implantation and post-natal development. Hence, the evaluation of the epigenetic status of ART embryo, especially blastocyst, as the final in vitro product of ART, would be of principal importance. Therefore, in this study, we assessed the methylation status of H19/IGF2 DMR in intra cytoplasmic sperm injection-derived blastocysts donated from healthy couples enrolled in ART program for family balancing.

Our results showed that the percentages of overall methylated CpGs and hyper-methylated clones of H19/IGF2 DMR in blastocysts were 37.85±4.87% and 43.75±5.1%, respectively, while this value for lymphocytes was 49.52±1.86% and 50%. The values obtained for lymphocytes was relatively similar to those reported previously in literature (the published mean methylation indices of 53.3±3.1%[13], 52.07±6.59%[31], 50±3.0%[32] and 49.8%[24]). Likewise, when defined the overall methylation of CpGs of H19/IGF2 DMR in blastocysts, we observed that the methylation status of the H19 DMR is lower than the expected methylation level of somatic cells (Fig. 2). This observation, to a certain degree, is in concordance with a previous report from the Lefèvre group[21]. Using genome-wide analysis of DNA methylation of early human blastocyst embryo, Okae et al.[33] also reported an average of methylation levels with a median of around 40% for gDMRs, which is close to the value reported in this study and Lefèvre group’s study[21]. It is important to note that Okae and colleagues[33] did not specifically report the methylation level of ICR1. To our knowledge, there is no other study to assess the DMR in high-quality human blastocysts with specific attention to gDMRs or ICR1. Therefore, the values obtained in this study and those reported by others[21,33], which are close to each other, can be considered as reference value for future studies.

In contrast to our results and the aforementioned studies[21,33], Chen et al.[27] reported higher degree of DNA methylation (49.4±9.7% as compared to our value 37.85±4.87%) in human embryos. However, the main difference between our study and Chen et al.,

Table 3. Average methylation for each clone in a representative replicate located in ICR1 sequence in the blastocyst.

| Seq. identifier | Average (%) |
|-----------------|-------------|
| 1               | 100         |
| 2               | 66.6        |
| 3               | 66.6        |
| 4               | 94.4        |
| 5               | 88.8        |
| 6               | 88.8        |
| 7               | 77.7        |
| 8               | 0.0         |
| 9               | 0.0         |
| 10              | 0.0         |
| 11              | 0.0         |
| 12              | 0.0         |
| 13              | 0.0         |
| 14              | 0.0         |
| 15              | 5.5         |
| 16              | 11.1        |

The number of clones is shown in the raw of “Seq. identifier”. Seq. sequence

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The present study show that the mean methylation status of ICR1 of the human good-quality blastocyst donated by healthy individuals is around 37.85±4.87%. However, since there is no comparison with in vivo embryos in human, it is not possible to conclude that the methylation found in the CpG is actually normal or abnormal. Furthermore, ART procedures should be optimized in order to at least reduce some of the risks associated with ART.

CONFLICT OF INTEREST. None declared.

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