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H19/Igf2 Expression and Methylation of Histone 3 in Mice Chimeric Blastocysts

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Abstract

Background: Currently, the efficient production of chimeric mice and their survival are still challenging. Recent researches have indicated that preimplantation embryo culture media and manipulation lead to abnormal methylation of histone in the *H19/Igf2* promotor region and consequently alter their gene expression pattern. This investigation was designed to evaluate the relationship between the methylation state of histone H3 and *H19/Igf2* expression in mice chimeric blastocysts.

Methods: Mouse 129/Sv embryonic stem cells (mESCs) expressing the green fluorescent protein (mESCs-GFP) were injected into the perivitelline space of 2.5 days post-coitis (dpc) embryos (C57BL/6) using a micromanipulator. H3K4 and H3K9 methylation, and H*19* and *Igf2* expression was measured by immunocytochemistry and q-PCR, respectively, in blastocysts.

Results: Histone H3 trimethylation in H3K4 and H3K9 in chimeric blastocysts was significantly less and greater, respectively ($p< 0.05$), than in controls. *H19* expression was significantly less ($p< 0.05$), while *Igf2* expression was less, but not significantly so, in chimeric than in control blastocysts.

Conclusions: Our results showed, that the alteration ofH3K4me3 and H3K9me3 methylation, change *H19/Igf2* expression in chimeric blastocysts.

Keywords: Chimeric blastocysts, *H19/Igf2*, Histone 3 (H3) methylation.

Introduction

Chimeras are animals composed of two or more genetically different cell lineages or recipient embryos from the same or different species (1). Chimeric animals can give insights into the biological processes in the adults, including mechanisms underlying diseases or regenerative medicine (2-4). Currently, injection of embryotic stem cells (ESCs into the blastocysts is the most common technique to generate chimera mice (1). Previous studies showed that microinjection of ESCs into the blastocyst is an efficient approach to produce a good germ line-transmitted chimera (5-7). In this regard, application of laser technology to help introduce ESCs into the perivitelline embryonic space has its own advantage to produce ESC-derived F0 chimaeras (1). Despite the advantages of chimeric mice in biological studies, the efficiency of generating and survival are still low.

Many studies have shown that manipulation of preimplantation embryos can cause birth defects including low birth weight, cardiovascular defects, congenital malformations, and abnormal placentation (8).

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Moreover, embryo manipulation and culture conditions can change early embryo development and gene expression patterns by modifying epigenetic factors (9). However, micromanipulation of embryos increases non-physiological epigenetic profiles that lead to aberrant chromatin remodeling and genomic imprinting, which can result in genetic diseases including Beckwith–Wiedemann and Angelman syndromes (10-12).

Evidence indicates that some imprinting genes are predominantly expressed by the maternal, while others only by the paternal, chromosome (13). Currently, about 100 proteins encoded by imprinted genes have been identified in both mice and human genomes (14). In mice embryos, *H19/Igf2* imprinting genes have greater sensitivity to culture supplements and micromanipulations than other imprinted genes (15, 16). Also, *H19/Igf2* have an essential function in the control of embryo development, placental organization, and fetal growth (17). A previous study observed that the abnormal imprinting of the *H19/Igf2* genes arose from abnormal histone modifications and atypical DNA methylation at the imprinting control region (ICR) (18-20). Differential epigenetic modification at the ICR and upstream of the transcription site of the imprinting genes in the preimplantation embryo have been observed (21). For instance, lysine methylations restricted to the promoter of the imprinted loci are H3 lysine 9 trimethylation (H3K9me3) and H3 lysine 4 trimethylation (H3K4me3), which lead to inhibition and activation, respectively, of gene expression in imprinting genes (15). Recent studies have shown that embryo culture and manipulations, including cryopreservation, intracytoplasmic sperm injection (ICSI), and somatic cell nuclear transfer **(**SCNT) can lead to the abnormal histone methylation in promoter regions of the imprinting genes (22, 23). However, histone 3 methylation and *H19/Igf2* expression has not yet been investigated in chimeric mice.

Materials and methods

Animal and Chemical

The experimental animal model use for this study was created based on the guidelines of the Research Ethics Committee of Shahid Beheshti University of Medical Sciences. All reagents were obtained from Sigma Chemical (St. Louis, USA), unless otherwise mentioned. Male and female C57BL/6 mice were obtained from the Pasteur institute, Tehran, Iran and housed in 50% humidified and temperature-controlled rooms at 20–24 °C) on a 12-hour light-dark cycle. All animals had free access to water and food.

Experimental Groups

H3K9 and H3K4 H3 histone methylation and the relative expression of the H19 and *Igf2* imprinting genes and were evaluated in the blastocysts of the following experimental groups: i. *in vivo*-derived blastocysts (blastocyst/*in vivo* or control; n= 60), ii. blastocysts obtained from *in vivo*-derived morula (blastocyst/2.5 days' post coitum (dpc) embryo; n= 60 (, and iii. blastocysts obtained from *in vivo*-derived morula that had been subjected to subzonal mESCs injection (Blastocyst/chimeric; n= 60). Total embryos were collected and cultured from the three groups simultaneously at the same developmental stage.

Collection of 2.5 days-post-coitis (dpc) embryos

Female mice (n= 20 C57BL/6), 8-10 weeks old, were superovulated with an intraperitoneal (IP) injection of 7.5 IU of pregnant mare serum gonadotropin (PMSG; Folligon, Intervet); followed by 7.5 IU of human chorionic gonadotropin (HCG; Novarel, Pregnyl) 46 to 48 hours later. These mice were then mated with C57BL/6 male mice. Females with vaginal plugs were euthanized by cervical dislocation at 2.5 dpc time and these embryos collected by oviduct flushing (24), transferred in a 30 µl droplet of KSOM medium supplemented with amino acids (KSOMaa) (25), and incubated in a 37 °C, 5% CO_2, humidified atmosphere.

Subzonal Injection of GFP-ESCs

Mouse 129/Sv ESCs, labeled with GFP (GFP-mESCs), were used for the subzonal injection. This line has been used for follow-up to ensure that the embryonic stem cells have integrated in the inner cell mass for chimeric formation. The GFP-mESCs were cultured in knockout DMEM

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(Invitrogen) supplemented with 15% Knockout Serum (KOSR; Invitrogen, Gibco), 1% MEM non-essential amino acids (Gibco), 2 mM GlutaMAX, 100 mM β-mercaptoethanol (Gibco), 100 U/ml penicillin, 100 mg/ml streptomycin, 1000 U/ml mouse leukemia inhibitory factor (LIF, Pro Spec), 2% ES-FBS (ES Cell Qualified FBS), R2i (1 µM PD0325901 (Selleck, USA), and 10 µM SB431542 (Selleck, USA). The GFP-mESCs were cultured on 12-well plates coated with sterile 0.1% gelatin. The cells were trypsinized at 70% confluency to gain a solution of single-cell suspension and maintained in ES cell medium supplemented with 0.2 M HEPES (26).

Next, 2.5 dpc, a laser beam (150 FU; Prime Tech Ltd, Tsuchiura-shi) was applied to the embryos' thin zona pellucidae. The inner surface of a 20 µm diameter injection needle was rinsed with 10% polyvinylpyrrolidone (PVP) and 15 mESCs were injected into the embryos' (n= 60) subzonal spaces using a Narishige micromanipulator. The mESCs were injected in a medium containing 0.2 M sucrose. The GFP-mESC-injected embryos were cultured in KSOMaa and incubated in a 37 °C, 6.5% CO_2, humidified atmosphere for 24 hours until the blastocyst stage (1). To ensure chimeric blastocyst formation, these blastocysts were observed under a fluorescent microscope to evaluate the incorporation of mESCs-GFP into the ICM and selected for immunocytochemistry and Real Time PCR.

Immunofluorescence staining of H3K9me3 and H3K4m3

Trimethylation of H3K9 and H3K4 in chimeric blastocysts was visualized by immunocytochemistry as previously described (27). Briefly, the zona pellucida was dissolved by Tyrode's acid (Sigma T1788; pH: 2.5) for 30 sec at room temperature, fixed by 4% paraformaldehyde for 30 min at 4 ºC, and permeabilized by 0.3% Triton X-100 for nearly 1 h at 4 ºC. The solution was blocked with 2% BSA/PBS for 40 min at 25 ºC and then incubated overnight at 4 ºC with primary antibodies against H3K9me3 (Abcam, Cambridge, MA, USA) diluted 1:200 and anti-H3K4me3, also diluted 1:200 (Abcam, Cambridge, MA, USA) for 1 h at 25 ºC. After washing with PBS/PVA for 10 min, the blastocysts were incubated with the secondary antibody, goat F(ab')2 anti-mouse IgG H&L (PE/Cy5.5), at 1:500 in 2% BSA/PBS for 90 min at 37 ºC (Abcam, Cambridge, MA, USA). After 3 rinses, the blastocyst nuclei were stained by 15 µg/mL of 6-diamidino-2-phenylindole (DAPI) (CA, USA) for 10 min and all the samples were mounted on the slides with glycerol. Each assay was performed in triplicate and at least 40 blastocysts were analyzed for each group. The samples were evaluated through an epifluorescence microscope (Nikon, Tokyo, Japan) and immunofluorescence staining images obtained with a digital camera (HD1080p CMOS color camera, Euromex). The fluorescent images of the blastocysts were analyzed by the ImageJ software (Bethesda, MD).

Gene Expression by Real-Time PCR

RNA extraction

Total RNA was extracted from 5 blastocysts by TRIzol reagent (Life Technologies, Gent, Belgium) based on the manufacturer's instructions. Briefly, the blastocysts were homogenized in 50 µl of TRIzol, 25 µl of chloroform were added to each sample, kept at 25 °C (room temperature; RT) for 5 min, and centrifuged at 8000 g for 5 min at 4 °C. The RNA was precipitated by adding isopropanol and then centrifuged at 8000 g for 5 min. The supernatant was disposed, and the RNA washed with 80% ethanol. Total RNA was resuspended in 10 µl of DEPC water and stored at -80 ºC.

cDNA synthesis

The RNA concentration was determined by a spectrophotometer (Picodrop, Real-Life). Complementary DNA (cDNA) was produced using a PrimeScript QuantiTect Kit (Qiagen, cat: 205310). All reactions were performed in 20 µl volumes containing 2 µl of genomic DNA (gDNA), 13 µl of total RNA, 4 µl of enzyme buffer, and 1 µl of enzyme under the following thermocycling condition: 42 ºC for 2 min, 42 ºC for 15 min, and 95 ºC for 3 min. The cDNAs were stored at-20 °C until used in quantitative PCR.

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Quantitative reverse transcriptase PCR (qRT-PCR)

The relative expression of *H19* and *Igf2* was determined by qRT-PCR using a Rotor-Gene Q instrument (Qiagen). All reactions were performed in 10 µl volumes containing 5 µl of SYBR Premix Ex Taq II reagent (Takara Bio), 0.2 µl of each primer (10 µM), 2 µl of cDNA template, and 2.6 µl of ddH2O. The qPCR primers are listed in Table 1. The program used for PCR amplification was 95 °C for 30 sec as initiation, 50 cycles at 95 °C for 5 sec as denaturation, 60 °C for 30 sec as annealing/extension, and 60 to 95 °C with a ramp rate of 0.3 °C/s as the melting curve. GAPDH and H2AFZ were used as internal controls and the samples' mRNA levels were normalized against them. Three replicates were performed for each group. The relative mRNA expression was evaluated by REST 2009 Software (Qiagen, Hilden, Germany).

Table 1. Primers used for real-time quantitative PCR.

	Primer sequences (5′-3′)	**Tm**
Gapdh	Forward: TTCCAGTATGATTCCACCCAC Reverse: ACTCAGCACCAGCATCACC	55.9 55.7
H2afz	Forward: CTCGTCTCTTCCTCGCTCGT Reverse: CGTCCGTGGCTGGTTGTC	61.3 61.04
Igf2	Forward: TGTGAGCAAGCGACGGAGT Reverse: GGATTCAGTGGCTGGCAGA	58.3 58.6
H19	Forward: TGAAGGCGAGGATGACAGGT Reverse: TCCAGAGAGCAGCAGAGAAGTG	58.9 60

Statistical Analysis

The fluorescent intensity of histone methylation was analyzed using one-way ANOVA test (Tukey's post-hoc) and expressed as mean ± SD. Analyses were performed using the SPSS statistical software, version 19 (Armonk, NY, USA). Differences were considered to be statistically significant at $p< 0.05$.

Results

Immunocytochemistry

We used the mESCs carrying a GFP repoter to allow the chimeric blastocysts monitoring in the integration or exclusion during of the chimaera formation (Fig. 1.A). The methylation of H3K4 and H3K9 in the blastocysts were visualized by immunocytochemistry (Fig. 1) and processed with using of Image J software. The levels of methylation were evaluated by antibodies against H3K4me3 (red) and H3K9me 3 (red). The DNA is counterstained with DAPI (blue) and the merged images of H3K4me3 and H3K9me3 with DNA are shown purple (Fig. 1). While no significant difference was seen between the two groups blastocyst/*in vivo* and blastocyst/2.5 (dpc) embryo, fluorescence intensity in the H3K4 chimeric blastocysts was significantly less than that of the other two groups, ($p< 0.05$, Fig. 2A).

In contrast, fluorescence intensity in the H3K9 blastocyst/2.5 (dpc) embryo was significantly greater than of blastocyst/*in vivo*, also, fluorescence intensity in the H3K9 chimeric blastocyst was significantly greater than that of other two groups ($p< 0.05$, Fig. 2A).

These results indicate that there are dramatic reduction and increasing histone H3 methylation in H3K4 and H3K9 during chimeric blastocysts production, respectively.

Quantitative Gene Expression

H19 expression was significantly less in chimeric blastocysts than of other two groups ($p< 0.05$, Fig. 2B), while no significant difference was observed between blastocyst/*in vivo* and blastocyst/2.5 dpc embryo. In contrast, *IGF2* expression did not differ significantly between any of the 3 groups ($p> 0.05$, Fig. 2B).

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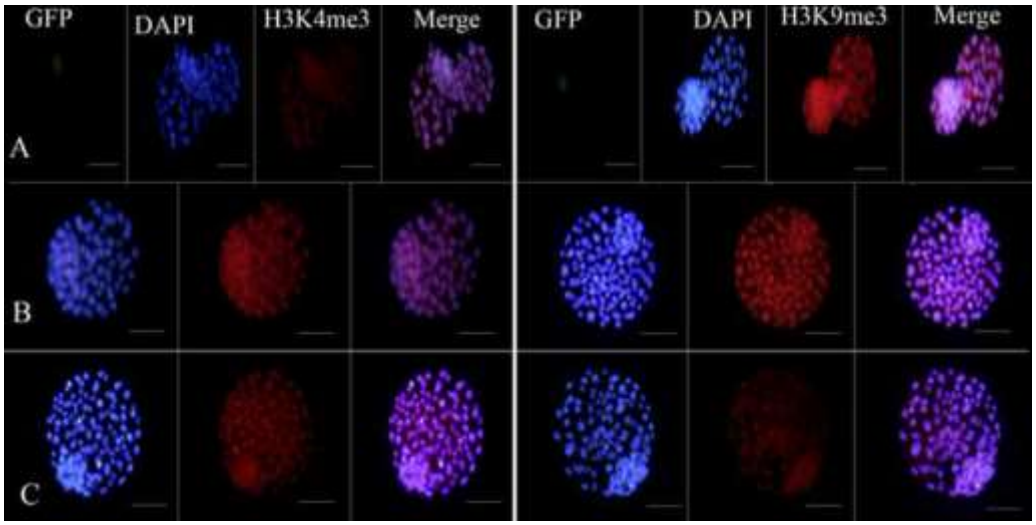

Fig. 1. Immunocytochemistry staining of H3K4me3 and H3K9me3 in chimeric blastocysts and blastocysts derived from other groups: (A): (Blastocyst/chimeric); B, Blastocyst/2.5 dpc embryo; C, Blastocyst/*in vivo*. The mESCs carrying green fluorescent protein (mESCs-GFP) marker that allows all the chimeric blastocysts to be analyzed for integration (GFP, Green). The nuclei (blue) were stained with DAPI. H3K4me3 and H3K9me3 protein were stained with using anti-mouse IgG (red). The merged images of H3K4me3 and H3K9me3 with DNA are purple. Scale bars: 50 μm.

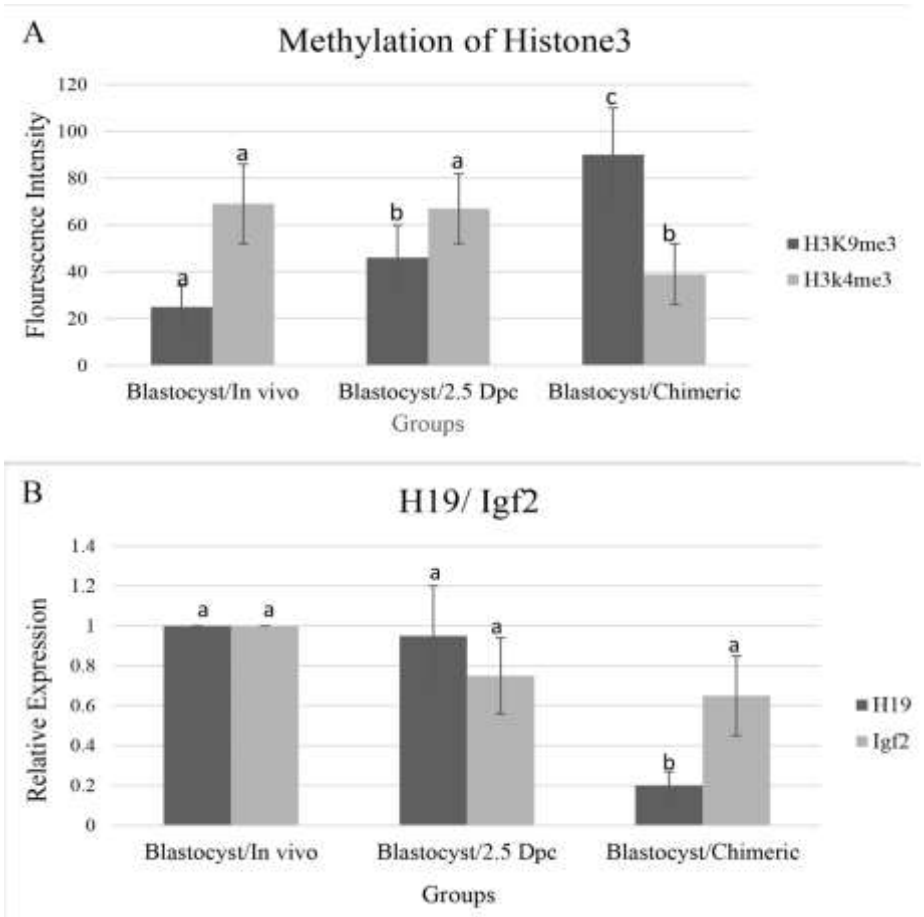

Fig. 2. Fluorescent intensities and relative gene expression in blastocysts; (A) H3K4me3 and H3K9me3 fluorescence intensity levels: Relative levels of global H3K9me3 and H3K4me3 in nucleus (mean ± SEM). a/b values with different superscripts differ significantly ($p< 0.05$) in relative levels of histone methylation were examined by Image-J software.; (B) *H19* and *Igf2* expression in the different blastocysts (Blastocyst/chimeric, Blastocyst/2.5 dpc embryo, Blastocyst/*in vivo*. Lower case letters a, b, and c above the bars represent statistical differences ($p< 0.05$). Values are shown as means ± SDs.

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Discussion

In vitro manipulation of embryo and culture conditions may lead to an increased risk of birth defects (8). Recent studies have shown that *in vitro* manipulations have a strong influence on embryo development and blastomeric arrangement (28). Embryo culture conditions can also affect preimplantation embryo quality as well as gene expression patterns through genetic and epigenetic modifications (29). Recent investigations showed that assisted reproductive technology (ATR) may lead to abnormalities in imprinting patterns that eventually lead to disorders in the fetus (30, 31).

Both *H19* and *Igf2* are critical regulatory genes involved in embryo development and morphology, feto-placental growth, and postnatal behavior (32). The maternally-expressed allele of *H19* is located in the imprinting regions of chromosomes 11 and 7 in humans and mice, respectively (33). Previous studies have demonstrated that *H19* expression is influence by *in vitro* manipulations such as *in-vitro* fertilization (IVF) and SCNT (10).

This study assessed the effect of histone 3 methylation on *H19* and *Igf2* expression and showed a decrease in *H19* expression in chimeric blastocysts relative to controls. This might be due to epigenetic alterations in these genes' ICR. In agreement with our results, Khosla et al. demonstrated that embryo fertilization and specific medium (M16 medium) down-regulated *H19* expression (34). Other studies indicated that IVF and Whitten's and KSOM media down-regulated *H19* expression (35). However, Jahangiri et al. reported that vitrification of two-cell stage embryos had no effect on *H19* and *MEST* expression patterns (15). Nonetheless, most recent studies have demonstrated that some types of manipulation and embryo media may alter imprinting gene expression through DNA methylation and histone modifications (36).

It has been demonstrated that trimethylation of H3K4 and H3K9 change the activation of chromatin in the preimplantation embryo (37). It is also known that the *H19* ICR has some methylation at histone H3 of H3K4 and H3K9 (35), so embryo micromanipulation and culture conditions can alter H3K9me3 and H3K4me3 methylation status in the *H19* ICR (38).

H3K4me3 has been enriched in the unmethylated allele of imprinting gene (39) and this was associated with transcription activation. H3K4me3 is commonly enriched around the promoter sites that lead to activation of specific genes (40). In our study, H3K4 methylation in chimeric blastocysts decreased relative to the control groups; consequently, *H19* expression was reduced in chimeric blastocysts. This result agrees with a study indicating that manipulation of *in vitro*-derived embryos can alter H3K4me3 methylation and subsequent expression of imprinting genes (41).

H3K9me3 that has been enriched in the methylated allele of imprinting gene is generally correlated with inactivation and reduced gene expression and heterochromatin formation (42, 43). In our study, we found a reverse relationship between H3K9me3 and *H19* expression in chimeric -blastocysts. Interestingly, the up-regulation of H3K9me3 decreased *H19* expression in chimeric blastocysts relative to the *in vivo* obtained counterparts. Therefore, it seems the reduced *H19* expression in chimeric blastocysts occurred due to down- and up-regulation of H3K4me3 and H3K9me3 in their ICR site, respectively.

Igf2 is expressed specifically by the paternal allele under balanced conditions, so that *H19* for normal regulation of fetal and placental development is related to *Igf2* expression (44). Li et al. reported that supplemental media and *in vitro* fertilization lead to abnormalities in the DNA and histone methylation sites in the *Igf2/H19* ICR (19). In the current study, despite decreased *Igf2* expression in chimeric blastocysts, its expression was not significantly different between the *in vivo*-derived blastocysts. This agrees with a study by Khosla et al. (35), in which manipulation and supplemental culture media changed *Igf2* expression in mouse preimplantation embryos (35). Also, *Igf2* expression in IVF, cloned, and vitrified blastocysts was less than that of *in vivo*

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blastocysts (12,45). It should be noted, despite the reduced expression of *Igf2* in most previous studies, in our study *Igf2* expression in chimeric blastocysts was not significantly different from that of *in vivo*-derived blastocysts. Therefore, according to our study, it is likely that *Igf2* expression has been influenced by various factors including embryo micromanipulation, interaction of injected mESCs with embryo blastomeres, and supplementation of culture medium with BSA and non-essential amino acids. In this regard, in the chimeric blastocysts, down- and up-regulation of H3K4me3 and H3K9me3 in the ICR may be related to the reduced *H19* and partially-reduced *Igf2* expression.

In this study, the chimeric blastocysts had abnormal H3K9me3 and H3K4me3 levels, which led to reduced expression of the imprinting genes *H19* and *Igf2*, with *H19* expression being significantly more decreased than *Igf2*.

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