Int J Clin Exp Pathol 2016;9(4):4862-4871
www.ijcep.com /ISSN:1936-2625/IJCEP0023697

Original Article

Tissue-specific differential DNA methylation at *Igf2/H19* locus in a mouse model for embryonal ethanol exposure

Jizi Zhou[1*], Yanyan Qian[2,3*], Qiongjie Zhou[1], Yu Xiong[1], Huijun Wang[3], Duan Ma[2,3,4], Xiaotian Li[1,4,5]

[1]Obstetrics and Gynecology Hospital, Fudan University, Shanghai, China; [2]Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, Institute of Bio Medical Sciences, Shanghai Medical College, Fudan University, Shanghai, China; [3]Children's Hospital, Fudan University, Shanghai, China; [4]Institutes of Biomedical Sciences, Fudan University, Shanghai, China; [5]Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai, China. [*]Co-first authors.

Received January 10, 2016; Accepted March 22, 2016; Epub April 1, 2016; Published April 15, 2016

Abstract: Objective: Maternal ethanol consumption during pregnancy could induce birth defects. Fetal heart, brain and placenta are usually targeted organs. Differential methylation regions (DMRs) in the *Igf2/H19* locus are sensitive to environmental conditions. However, the effects of prenatal ethanol consumption on fetal heart, brain and placenta remain largely unknown. The aim of our study is to investigate tissue-specific effects of ethanol consumption during pregnancy on DNA methylation within *Igf2/H19* DMRs and the expression of Igf2 in fetal mouse heart, brain, and placenta. Methods: We established a pregnant mouse ethanol gavage model (0.025 ml/g/day; 25% vol:vol). Ethanol was delivered between gestational day (GD) 0.5 and 15.5. Using a MassARRAY EpiTYPER assay, methylation patterns of 4 DMRs, including *Igf2* DMR0, *Igf2* DMR1, *Igf2* DMR2, and *H19* DMR, within the *Igf2/H19* locus were evaluated in fetal tissues. In parallel, the expression of insulin-like growth factor (*Igf2*) gene was measured by qRT-PCR and immunohistochemistry. Results: We found decreased DNA methylations at *Igf2* DMR1, *Igf2* DMR2, and *H19* DMR in response to ethanol exposure in murine fetal heart, brain, and placenta, respectively. However, no significant methylation changes were observed at *Igf2* DMR0 in all above examined tissues. Importantly, we detected increased Igf2 expression at mRNA and protein levels in corresponding fetal mice brain in response to ethanol exposure. Moreover, for ethanol-treated group, decreased Igf2 expression in fetal mice heart and increased Igf2 expression in fetal mice placenta at mRNA and protein levels were observed compared to controls, although the changes showed no significant differences. Conclusion: Our data indicates that the underlying mechanisms by which fetal developmentally important imprinting genes are dysregulated in a tissue-specific fashion in response to maternal ethanol exposure.

Keywords: DNA methylation, DMRs, *Igf2/H19* locus, maternal ethanol consumption, fetal mice

Introduction

Maternal ethanol consumption during pregnancy is a worldwide public health issue, occurring in 20% of all pregnancies [1]. Ethanol consumption during pregnancy is teratogenic, adversely affecting the development of fetal brain and heart, and potentially leading to neurobehavioral abnormalities [2] and cardiac defects [3]. In addition to disrupting normal growth of fetal organs, the placenta is another target of ethanol. Prenatal ethanol consumption by the mother not only changes placental weight [4], but also disrupts the placental detoxification mechanisms as well as nutrient distribution throughout the placenta [5, 6]. Fetal brain, heart, and placenta are uniquely affected by ethanol, but the individual molecular mechanisms behind these effects are unclear.

In mammals, a small subset of imprinting genes are considered key to fetal growth and development, particularly for neurologic behavior, and placental development [7, 8]. Imprinting genes are usually clustered in the genome under the control of differentially methylated regions (DMRs), which are normally methylated during gametogenesis [9]. Aberrant DNA methylation at DMRs is implicated in imprinting diseases and developmental abnormalities [10].

Among imprinting genes, *Igf2* gene has been widely studied in many tissues including the heart, brain and placenta. Igf2 is expressed virtually throughout the brain and is essential to central nervous system development [11]. In *in vivo* experiments, Igf2, expressed in the embryonic mouse epicardium during midgestational heart development, is required for normal ventricular chamber development [12]. Moreover, Igf2 is important for placental growth [13]. Interestingly, imprinted gene *Igf2* expression can be regulated by methylation of DMRs in the *Igf2/H19* imprinting region [14], located distally on mouse chromosome 7 [15]. A total of 4 DMRs exist in the *Igf2/H19* region [16]: DMR0, DMR1, DMR2 and *H19* DMR. Delicate DNA methylation control of the Igf2/H19 locus is important for pre- and post-natal growth and disruptions of methylation at the Igf2/H19 locus have been reported in congenital growth disorders and cancers [10].

Early *in vitro* studies suggest that environmental exposures affect the epigenetic regulation of genomic imprinting and are associated with aberrant fetal growth and development [17]. Additionally, previous studies suggest that methylation of one or some DMR(s) in the *Igf2/H19* region were sensitive to specific *in utero* exposures and postnatal diets [18, 19]. To date, the effect of prenatal ethanol exposure on methylation in the Igf2/H19 region has been confined to reproductive systems or fetal blood DNA [20, 21]. However, effects of *in utero* ethanol exposure on DNA methylation at *Igf2/H19* DMRs and their association with imprint gene expression in different fetal organs, such as heart, brain, and placenta, are not well-characterized. Given that DNA methylation and gene expression are highly regulated in tissue- or cell type-specific manner [22] in response to developmental and environmental cues, we hypothesized that prenatal exposure to ethanol could cause alterations of *Igf2/H19* DMRs methylation pattern and related Igf2 expression in tissue-specific manner in mouse model.

Materials and methods

Mice and tissue collection

C57BL/6J mice (weighing approximately 20-25 g) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd and raised in the animal facility at Shanghai Medical College, Fudan University. Mice were kept on a 12-hour light/dark cycle and were given free access to food and water at a constant room temperature. Then, 8-10 week-old female mice were naturally mated with same-aged male mice. Validation of a vaginal plug the next morning after presumed mating was observed as gestational day 0.5 (GD 0.5). Plug-positive female mice were separated into 2 groups (N=3, each): one group was given 500 μl ethanol by gavage (0.025 ml/g/day; 25% vol: vol), a dose recommended in a previous study [23]; the second group received an equal volume of distilled water between GD 0.5 and 15.5. Ethanol administration was coincident with a key period of fetal organogenesis and development [24]. Pregnant mice were sacrificed on GD 16.5. The heart, brain, and placenta tissues from 17 fetal mice of the control group and 16 fetal mice of the ethanol-treated group were analyzed for methylation. The heart, brain, and placenta tissues from 12 fetal mice in the ethanol-treated group and from 11 fetal mice in the control group were analyzed for Igf-2 expression at mRNA and protein level. To homogenize the brain tissues, we tried to dissect the cerebral cortex part. All tissues for methylation and mRNA expression analysis were excised under a dissecting microscope and immediately placed in RNAlater® (AMBION, Inc., Austin, TX) for at least 24 hours at room temperature, and then frozen at -80°C until use. All procedures were approved by the Committee for the Care and Use of Laboratory Animals of Fudan University.

DNA isolation

Total genomic DNA was extracted from heart, brain, and placenta using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and purity were measured by absorbance of A260/A280 ≥ 1.8 and A260/A230 ≥ 1.9 using a NanoDropTM1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware).

Bisulfite treatment

An EZ DNA Methylation Kit™ was used to perform sodium bisulfite treatment for the extracted DNA according to manufacturer's instructions (Zymo Research, Orange, CA). Sodium bisulfite converted DNA was re-suspended in

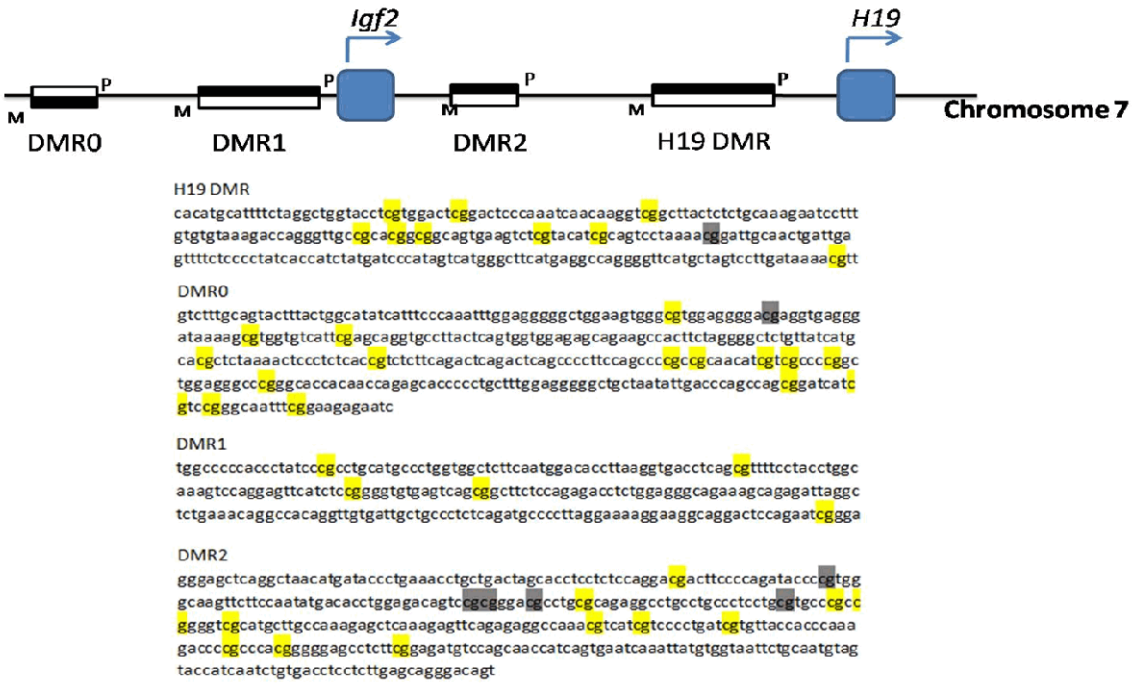

Figure 1. Schematic diagram of Igf2/H19 locus. The sequences detected in each DMR are listed. Yellow highlighting represents CpG in detectable sites, gray highlighting represents undetectable sites.

10 ml elution buffer and stored at -20°C until samples were used for experiments.

Massarray epityper assay

The Sequenom MassARRAY EpiTYPER platform was used to measure methylation of DMRs within the *Igf2/H19* locus. The EpiTYPER assay can quantify cytosine phosphate guanine (CpGs) dinucleotide methylation based on MALDI TOF mass spectrometry, which is an accurate, sensitive and high-throughput method for quantitative analysis of DNA methylation at CpG sites [25]. Sequences of analyzed DMRs within Igf2/H19 have been described elsewhere [26]. Specific CpG sites and their location are depicted in **Figure 1**. Primers used in this study were designed online (http://epidesigner.com; **Table 1**). Approximately 500 ng fragmented DNA from each sample was modified by bisulfite treatment and this was followed by PCR with specific primers, which add a T7 promoter tag for the reverse primer and a 10-mer tag for the forward primer. Then, PCR products were treated with shrimp alkaline phosphatase. After ligating fragments to a T7 promoter segment, they were transcribed into RNA. Synthesized RNA was cleaved with Rnase A and all cleavage products were analyzed using Epityper software (version 1.0; Sequenom). The detailed protocol for this process has been published previously [27].

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA of fetal mice tissue was extracted with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) from 100-200 mg of frozen tissue. Approximately 500 ng of the extracted RNA sample was used as a template for the reverse transcriptase reaction. Transcribed cDNA was amplified in a qPCR reaction using Sybr Green (TAKARA Biotechnology, Dalian, Liaoning, China), and analyzed with an Applied Biosystems 7900 real-time PCR system. Gene expression comparisons were conducted for the *Igf2* gene (**Table 2**). All assays were performed in triplicate. *Gapdh* was used as a housekeeping gene for normalization and data were analyzed with the CT method after averaging the triplicates of each assay.

Immunohistochemistry analysis

Heart, brain, and placental tissue were fixed in 4% formalin overnight and then embedded in

Table 1. Primers used for DNA methylation of 4 DMRs at the Igf2/H19 locus

Primers	Forward 10-mer tag primera1	Reverse T7 promoter tag2	Product size (bp)	Assayed CGs (n)
H19 DMR	ATAACCCCAAAACCCTATAAATCAA	GGGGTTATAAATGTTATTAGGGGG	323	9
IGF2 DMR0	TCACTAATACCCAAAATTCTCTTCC	TATTATTTTTTAAATTTGGAGGGGG	339	10
IGF2 DMR1	AAAAACACATACATACCCTAACCCC	GTTAGAGATGAGTAAGGTTTTGGGA	292	5
IGF2 DMR2	TCAAACTAACATAATACCCTAAAACC	TGGAATTGTTTTTGTTTAAGAGGAG	359	12

110-mer tag: cagtaatacgactcactatagggagaagg and 2T7 promoter tag: aggaagagag were added.

Table 2. Primers used for qRT-PCR analysis of Igf2 gene expression

Primers	Forward	Reverse
Mouse *Igf2*	CCCGGAGAGACTCTGTGCGGA	GGAAGTACGGCCTGAGAGGTA
Mouse *GAPDH*	GGCAAATTCAACGGCACAGT	AGATGGTGATGGGCTTCCC

paraffin and 5-μm sections were made. Sections were dewaxed in xylene (3 times, 7 min for each incubation) and rehydrated in graded ethanol baths (twice in ethanol for 5 min each, once in 85% ethanol for 5 min and once in 75% ethanol for 5 min). Primary and second antibodies were diluted in 10% BSA (1:1,000). Anti-Igf2 (Abcam #ab9574) was used for staining. For Igf-2, the brown area on the section was selected for its color range. The staining was evaluated using the immunoreactive score (IRS) system described by Remmele and Stegner [28]. The staining intensity was scored as +, ++, +++ and the percentage of positively-stained cells was scored from 1 to 4.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Because of the small sample size, we assumed that data were not normally distributed. The non-parametric Mann-Whitney U test was used to compare methylation values, Igf2 expression at mRNA level and protein level between the ethanol-treated group and the control group. Statistical significance was set at 0.05.

Results

DNA methylation level at 4 DMRs in the Igf2/H19 locus in fetal mice heart, brain and placenta

To investigate the effect of maternal ethanol consumption on DNA methylation at *Igf2/H19* locus in fetal mice heart, brain and placenta, we focused on 4 DMRs (**Figure 1**): DMR0, DMR1, DMR2 and *H19* DMR. By using Sequenom MassARRAY EpiTYPER platform, 2-5% lower methylation was found in ethanol exposed fetal mice tissues. For fetal mice heart, there was 5% decreased DNA methylation at the DMR1 rather than the other three DMRs, which was statistically different compared to the control group (P=0.01, **Figure 2A**). To further analyze individual CpG site within DMR1, the methylation level at CpG4 and CpG5 sites were found to be statistically significantly decreased (P=0.015, P=0.0064, **Figure 2A**). For fetal mice brain from ethanol-treated group, using the same method, we detected that significantly decreased methylation only occurred at the DMR2 (P=0.0018, **Figure 2B**). Among 16 CpG sites within DMR2, the methylation level at CpG8.9, CpG11 and CpG16 sites was significantly decreased (P=0.0243, P=0.0007, P=0.0191, **Figure 2B**). Similarly, in fetal mice placenta, the H19 DMR was the only region with altered methylation, which was significantly decreased in the ethanol-treated group (P=0.0003, **Figure 2C**). Among the 10 CpG sites analyzed, the methylation level of CpG3, CpG4, CpG5.6, CpG7 and CpG8 sites was significantly decreased (P=0.0173, P=0.0023, P=0.0031, P=0.0087, P=0.0479, **Figure 2C**). However, no significant methylation changes were observed at *Igf* DMR0 in all above examined tissues. Taken together, these results suggested that DNA methylation of four DMRs change with a tissue-specific pattern in fetal organs in response to maternal ethanol exposure.

Igf2 mRNA and protein expression in fetal mice heart, brain, and placenta in response to maternal ethanol exposure

To investigate whether maternal ethanol exposure influenced Igf2 expression, mRNA level of

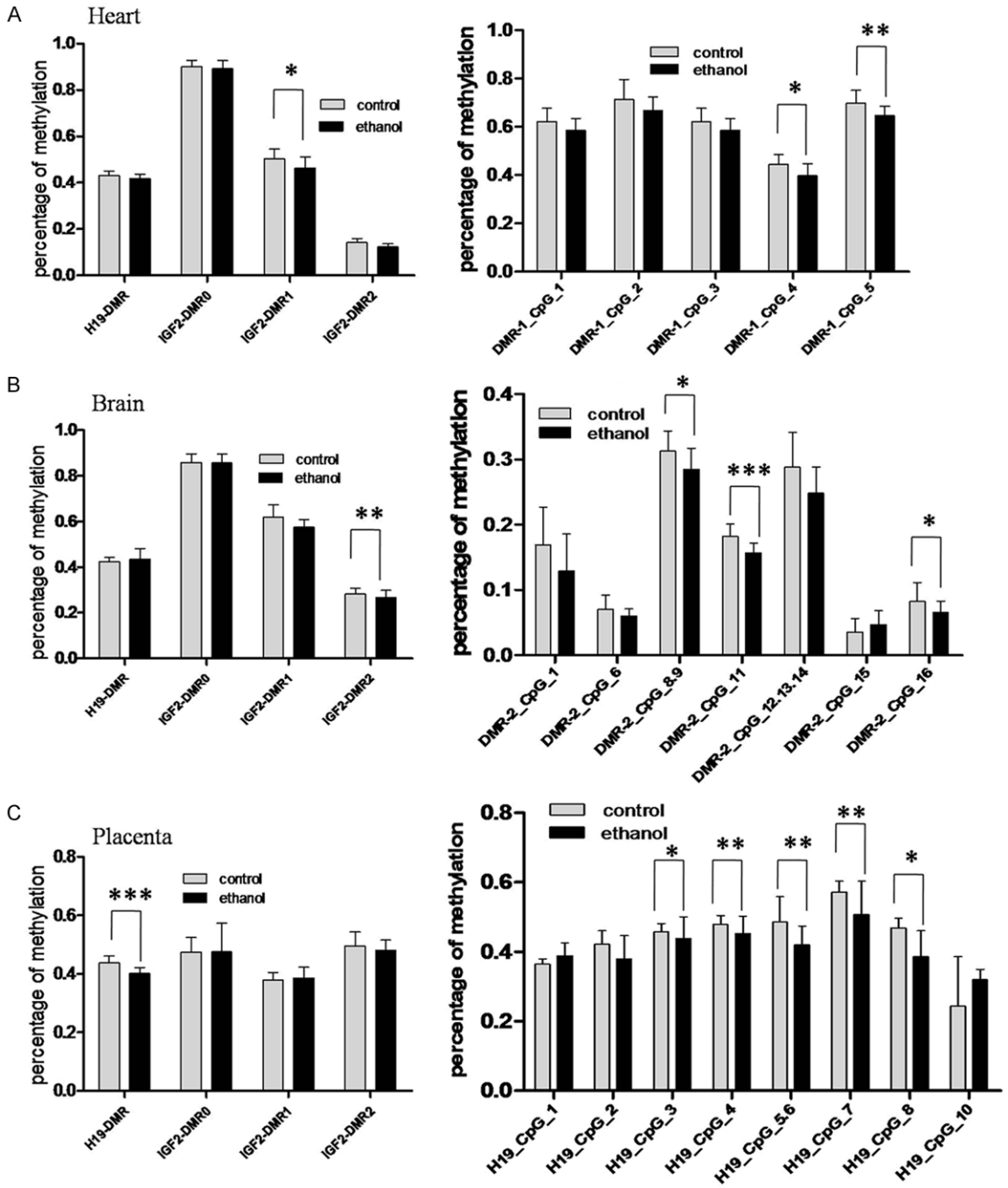

Figure 2. Comparisons of methylation of *H19* DMR, *Igf2* DMR0, *Igf2* DMR1, *Igf2* DMR2 in fetal mouse hearts (A), brains (B) and placentas (C) comparing the ethanol-exposed group to the control group. The left panel of the figure depicts average methylation of 4 DMRs. Detailed methylation in each CpG site in each DMR region is shown on the right panel. *P<0.05, **P<0.01, ***P<0.001.

GD16.5 fetal mice hearts, brains, and placentas were assayed by RT-qPCR. And we found that *Igf2* mRNA expression in the brains of ethanol-treated fetal mice statistically significantly increased compared to the control group (P=0.0121, see **Figure 3**). *Igf2* mRNA expression had a 53% decrease in the hearts of ethanol-treated fetal mice, but a 55% increase in the placentas, compared to the control group. However, neither of them was statistically sig-

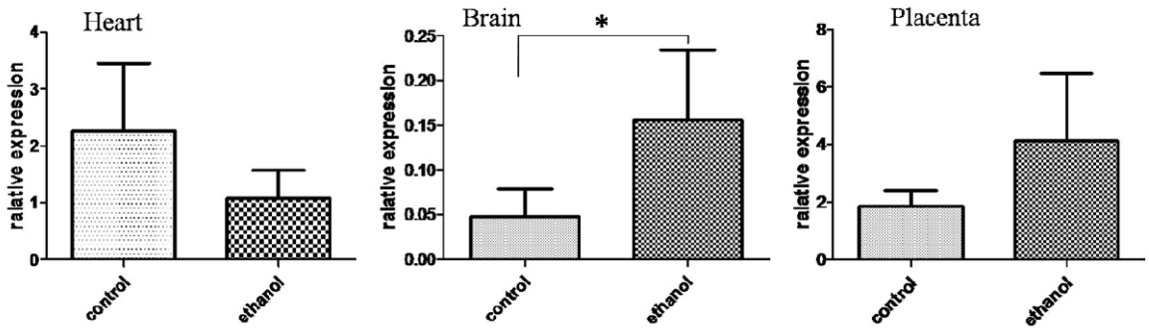

Figure 3. Igf2 expression at the mRNA level as measured with qRT-PCR in fetal mouse hearts, brains, and placentas between the ethanol-treated (n=12) and control groups (n=11). *P<0.05.

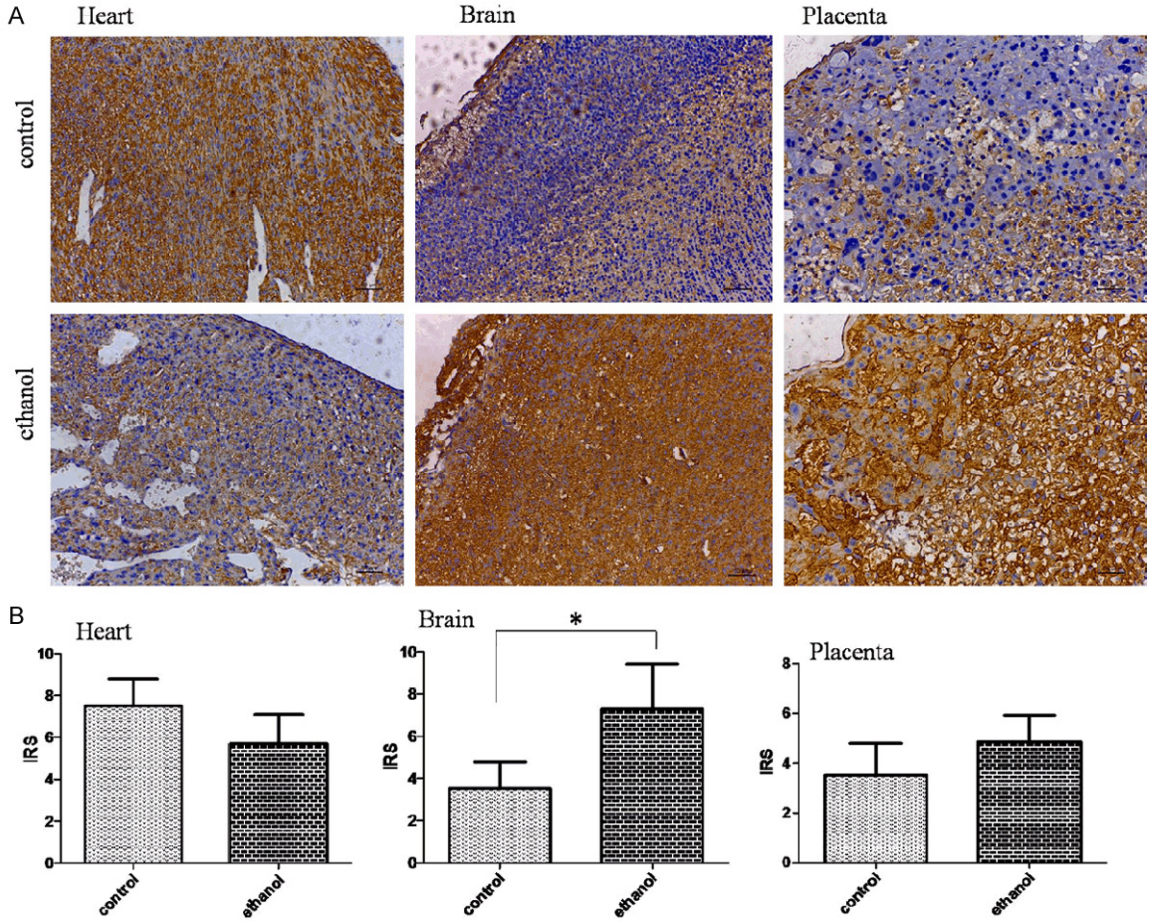

Figure 4. A. The examples from immunohistochemical results in fetal mice heart, brain, and placenta between the ethanol-treated and control groups. All images original magnification, ×200, and scale bar, 50 μm. B. The immunoreactive scores of Igf2 protein expression in fetal mice hearts, brains, and placentas between the two groups. See Materials and Methods for details on scoring systems. *P<0.05.

nificant (P=0.0727 and P=0.3152, see **Figure 3**).

Similar to the data for mRNA, Igf2 protein expression was decreased in fetal mice hearts, and increased in fetal mice brains and placentas in the ethanol-treated group. **Figure 4A** indicates that Igf2 was mainly expressed in the cytoplasm of myocardial cells in fetal mice heart. Igf2 expression in fetal mice brain was

distributed in the cortex. While, Igf2 was expressed in the deciduas layer fetal mice placenta. The protein levels of Igf2 expression were semiquantified with IRS (**Figure 4B**). We again observed a significantly difference of Igf2 protein expression in fetal mice brain between the ethanol-treated and control group (P= 0.029, see **Figure 4B**). Although an individual difference in the protein level was observed in the fetal mice heart and placenta from the ethanol-treated group, the two groups showed no significant difference according to Mann-Whitney test (P=0.0788 and P=0.12, see **Figure 4B**). Negative control subjects treated with no Igf2 primary antibody showed no positive staining.

Discussion

In the present study, we revealed, for the first time, that the molecular mechanisms by which maternal ethanol consumption during pregnancy adversely affected fetal development was in a tissue-specific manner. We observed the decreased DNA methylation at the three different DMRs including *Igf2* DMR1 in fetal mice heart, *Igf2* DMR2 in fetal mice brain, and *H19* DMR in fetal mice placenta following prenatal exposure to ethanol. And this could be related to the increased Igf2 expression in fetal mice brain and placenta, but decreased Igf2 expression in fetal mice heart. Our results suggested that DNA methylation in the *Igf2/H19* locus and the subsequent expression of Igf2 caused by *in utero* exposure to ethanol is tissue-specific and may contribute to distinct fetal toxicities. Additionally, different relationships between DNA methylation and Igf2 expression changes might suggest partially distinct mechanisms for Igf2 expression control in different tissues.

Ethanol exposure during fetal development has been reported to be linked DNA methylation in many previous studies. Maternal consumption of ethanol during pregnancy can result in a hypomethylation of fetal DNA due to decreased DNA methyltransferase activity [21]. This could, at least partially, account for our data which revealed decreased methylation at three DMRs (*Igf2* DMR1, *Igf2* DMR2 and *H19* DMR in the fetal mice hearts, brains and placentas, respectively).

Current understanding of the effect of ethanol exposure on methylation and expression of imprinting genes such as *H19* and/or *Igf2* has come from studies in the whole embryo [29]. Downing and colleagues [30] reported small decreases in DNA methylation at four CpG sites in DMR1 regions of the *Igf2* locus in embryos, but no significant decreases in methylation in the placentas were observed after prenatal ethanol exposure. In contrast, Haycock and le Ramsay [31] observed that DNA methylation at both paternal and maternal alleles in the *H19* imprinting control region were unaffected in embryos exposed to ethanol, but paternal alleles were significantly less methylated in ethanol-treated placentas. The embryo is a heterogeneous tissue and this alone may confound methylation analyses. However, in our study, we adopted more homogeneous and specific tissues including the heart, brain and placenta. Our data suggest that a tissue-specific methylation changes in three different organs. A significant decrease in methylation occurred within the Igf2 DMR1 in fetal mice hearts but not in brains or placentas from ethanol-treated groups. Decreased methylation in Igf2 DMR2 was only determined in fetal mice brains but not in the hearts or placentas. Methylation decreased in the H19 DMR in fetal mice placentas but not in the hearts or brains.

Usually, inverse correlations between methylation and expression are seen in many gene regulatory regions, but alterations in DNA methylation may up-regulate or down-regulate gene expression [32]. Our data revealed decreased methylation of DMR1 and a decreased Igf2 expression trend in fetal mice heart, a finding that agreed with Downing and colleagues who also reported small decreases in DNA methylation at the Igf2 DMR1 and approximately 1.5-fold decreases in methylation in embryos [30]. We observed that in fetal mice placental tissue decreased methylation of *H19* DMR and an increased expression trend of Igf2 occurred, which was supported by others' report that the *H19* DMR on the maternal chromosome is required for *Igf2* silencing [33]. Furthermore, we suggest that, in fetal mice brain, there is a statistically significant inverse correlation between methylation of *Igf2* DMR2 and Igf2 expression. On the contrast, Dejeux and coworkers [34] observed that a loss of methylation in the IGF2 DMR2 caused reduced expression of IGF2 in endocrine tumors of the digestive

tract. It might be attributed to different tissues used in the two studies.

In summary, we observed that maternal ethanol consumption during pregnancy induced a distinct change in methylation patterns within DMRs at the *Igf2/H19* locus, potentially influencing Igf2 expression in the fetal mouse heart, brain and placenta. These results suggest tissue-specific DNA methylation and gene expression changes in fetal organs in response to maternal ethanol exposure.

However, there are some limitations in our study. Igf2 expression was non-statistically significantly different in fetal mice heart and placenta, which might be due to the small sample size used in the expression study. Other explanation regarding the non-statistically difference was that the expression of Igf2, to some extent, was possibly unstable and variable. Previous report has demonstrated that IGF2 concentration was changing along with age in different organ systems following prenatal exposure [35]. Although the difference was non-statistically, the changing trend of Igf2 expression in our study was in accordance with this report in fetal mice heart and brain except placenta. But our results regarding Igf2 expression in fetal mice placenta was supported by a recent report by X. Joya and et al [36] demonstrating that ethanol exposure during pregnancy caused an increase in the secretion of IGF2 in placenta. However, further studies are needed to elucidate the underlying mechanisms behind the tissue-specific Igf2 expression regulated by DNA methylation at DMRs in the Igf2/H19 locus.

Acknowledgements

This work was supported by the National Basic Research Program of China (973 program) (2010CB529500) and the National Science Fund of China (81270712, 81300506, 81200449, 81200448). We gratefully acknowledge the financial assistance provided by the Science Foundation for Young Scholars of Shanghai (13ZR1452000), and National Key Basic Research Plan of China (973 Plan) (2015CB943300).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Duan Ma, Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, Institute of Bio Medical Sciences, Shanghai Medical College, Fudan University, 138 Yixueyuan Road, Shanghai 200032, China. Tel: 86-21-54237616; Fax: 86-21-54237135; E-mail: duanma@fudan.edu.cn; Dr. Xiaotian Li, Obstetrics and Gynecology Hospital Affiliated to Fudan University, 419 Fangxie Road, Shanghai 200011, China. Tel: 86-21-63455050-418; Fax: 86-21-63455090; E-mail: xiaotianli555@163.com

References

[1] Bhuvaneswar CG. Alcohol use during pregnancy: prevalence and impact. Prim Care Companion J Clin Psychiatry 2007; 9: 455.

[2] Jones KL. The effects of alcohol on fetal development. Birth Defects Res Part C Embryo Today Rev 2011; 93: 3-11.

[3] Carmichael SL, Shaw GM, Yang W, Lammer EJ. Maternal periconceptional alcohol consumption and risk for conotruncal heart defects. Birth Defects Res Part A Clin Mol Teratol 2003; 67: 875-878.

[4] Rout UK and Dhossche JM. Liquid-diet with alcohol alters maternal, fetal and placental weights and the expression of molecules involved in integrin signaling in the fetal cerebral cortex. Int J Environ Res Public Health 2010; 7: 4023-4036.

[5] Veid J, Karttunen V, Myöhänen K, Myllynen P, Auriola S, Halonen T, Vähäkangas K. Acute effects of ethanol on the transfer of nicotine and two dietary carcinogens in human placental perfusion. Toxicol Lett 2011; 205: 257-264.

[6] Hutson JR, Stade B, Lehotay DC, Collier CP, Kapur BM. Folic acid transport to the human fetus is decreased in pregnancies with chronic alcohol exposure. PLoS One 2012; 7: e38057.

[7] Ishida M and Moore GE. The role of imprinted genes in humans. Mol Aspects Med 2013; 34: 826-40.

[8] Frost JM and Moore GE. The importance of imprinting in the human placenta. PLoS Genet 2010; 6: e1001015.

[9] Lewis A and Reik W. How imprinting centres work. Cytogenet Genome Res 2006; 113: 81-89.

[10] Murrell A, Ito Y, Verde G, Huddleston J, Woodfine K, Silengo MC, Spreafico F, Perotti D, De Crescenzo A, Sparago A, Cerrato F, Riccio A. Distinct methylation changes at the IGF2-H19 locus in congenital growth disorders and cancer. PLoS One 2008; 3: e1849.

[11] Benarroch EE. Insulin-like growth factors in the brain and their potential clinical implications. Neurology 2012; 79: 2148-2153.

[12] Li P, IGF signaling directs ventricular cardiomyocyte proliferation during embryonic heart development. Development 2011; 138: 1795-1805.
[13] Constância M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C, Reik W. Placental-specific IGF-II is a major modulator of placental and fetal growth. Nature 2002; 417: 945-948.
[14] Ding GL, Wang FF, Shu J, Tian S, Jiang Y, Zhang D, Wang N, Luo Q, Zhang Y, Jin F, Leung PC, Sheng JZ, Huang HF. Transgenerational glucose intolerance with Igf2/H19 epigenetic alterations in mouse islet induced by intrauterine hyperglycemia. Diabetes 2012; 61: 1133-1142.
[15] Delaval K and Feil R. Epigenetic regulation of mammalian genomic imprinting. Curr opin Genet Dev 2004; 14: 188-195.
[16] Ollikainen M, Smith KR, Joo EJ, Ng HK, Andronikos R, Novakovic B, Abdul Aziz NK, Carlin JB, Morley R, Saffery R, Craig JM. DNA methylation analysis of multiple tissues from newborn twins reveals both genetic and intrauterine components to variation in the human neonatal epigenome. Hum Mol Genet 2010; 19: 4176-4188.
[17] Thompson SL, Konfortova G, Gregory RI, Reik W, Dean W, Feil R. Environmental effects on genomic imprinting in mammals. Toxicology letters 2001; 120: 143-150.
[18] Shin HS, Seo JH, Jeong SH, Park SW, Park Y, Son SW, Kim JS, Kang HG. Exposure of pregnant mice to chlorpyrifos-methyl alters embryonic H19 gene methylation patterns. Environ Toxicol 2014; 29: 926-35.
[19] Steegers-Theunissen RP, Obermann-Borst SA, Kremer D, Lindemans J, Siebel C, Steegers EA, Slagboom PE, Heijmans BT. Periconceptional maternal folic acid use of 400 μg per day is related to increased methylation of the IGF2 gene in the very young child. PLoS One 2009; 4: e7845.
[20] Ouko LA, Shantikumar K, Knezovich J, Haycock P, Schnugh DJ, Ramsay M. Effect of Alcohol Consumption on CpG Methylation in the Differentially Methylated Regions of H19 and IG-DMR in Male Gametes-Implications for Fetal Alcohol Spectrum Disorders. Alcohol Clin Exp Res 2009; 33: 1615-1627.
[21] Garro AJ, McBeth DL, Lima V, Lieber CS. Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. Alcohol Clin Exp Res 1991; 15: 395-398.
[22] Zille, MJ, Gu H, Müller F, Donaghey J, Tsai LT, Kohlbacher O, De Jager PL, Rosen ED, Bennett DA, Bernstein BE, Gnirke A, Meissner A. Charting a dynamic DNA methylation landscape of the human genome. Nature 2013; 500: 477-481.
[23] Webster WS, Germain MA, Lipson A, Walsh D. Alcohol and congenital heart defects: an experimental study in mice. Cardiovasc Res 1984; 18: 335-338.
[24] Serrano M, Han M, Brinez P, Linask KK. Fetal alcohol syndrome: cardiac birth defects in mice and prevention with folate. Am J Obstet Gynecol 2010; 203: 75, e7-75, e15.
[25] Ehrich M, Nelson MR, Stanssens P, Zabeau M, Liloglou T, Xinarianos G, Cantor CR, Field JK, van den Boom D. Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci U S A 2005; 102: 15785-15790.
[26] Waterland RA, Lin JR, Smith CA, Jirtle RL. Post-weaning diet affects genomic imprinting at the insulin-like growth factor 2 (Igf2) locus. Hum Mol Genet 2006; 15: 705-716.
[27] Sheng W, Wang H, Ma X, Qian Y, Zhang P, Wu Y, Zheng F, Chen L, Huang G, Ma D. LINE-1 methylation status and its association with tetralogy of fallot in infants. BMC Med Genomics 2012; 5: 20.
[28] Remmele W and Stegner H. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. Der Pathologe 1987; 8: 138.
[29] Liu Y, Balaraman Y, Wang G, Nephew KP, Zhou FC. Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation. Epigenetics 2009; 4: 500-511.
[30] Downing C,Johnson TE, Larson C, Leakey TI, Siegfried RN, Rafferty TM, Cooney CA. Subtle decreases in DNA methylation and gene expression at the mouse *Igf2* locus following prenatal alcohol exposure: effects of a methyl-supplemented diet. Alcohol 2011; 45: 65-71.
[31] Haycock PC and Ramsay M. Exposure of mouse embryos to ethanol during preimplantation development: effect on DNA methylation in the H19 imprinting control region. Biol Reprod 2009; 81: 618-627.
[32] Ehrlich M. Expression of various genes is controlled by DNA methylation during mammalian development. J Cell Biochem 2003; 88: 899-910.
[33] Thorvaldsen JL, Duran KL and Bartolomei MS. Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2. Genes Dev 1998; 12: 3693-3702.
[34] Dejeux E, Olaso R, Dousset B, Audebourg A, Gut IG, Terris B, Tost J. Hypermethylation of the IGF2 differentially methylated region 2 is a specific event in insulinomas leading to loss-of-

imprinting and overexpression. Endocr Relat Cancer 2009; 16: 939-952.

[35] Mauceri HJ, Lee WH andConway S. Effect of Ethanol on Insulin-Like Growth Factor-II Release from Fetal Organs. Alcohol Clin Exp Res 1994; 18: 35-41.

[36] Joya X, Salat-Batlle J, Velezmoro-Jáuregui G, Clavé S, Garcia-Algar O, Vall O. Prenatal ethanol exposure and placental hCG and IGF2 expression. Placenta 2015; 36: 854-62.