J Assist Reprod Genet (2015) 32:713–723
DOI 10.1007/s10815-015-0463-9

EPIGENETICS

Dysregulation of methylation and expression of imprinted genes in oocytes and reproductive tissues in mice of advanced maternal age

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Received: 4 December 2014 / Accepted: 6 March 2015 / Published online: 24 March 2015

Abstract
Purpose To evaluate reproductive outcomes in aged compared to young female mice, and determine associated methylation and expression of imprinted genes in reproductive tissues.
Methods Fetal, placental, and ovarian tissue were collected on d16.5 of pregnancy from young (4–5 weeks) and aged (15 months) mice. Uterine tissue and in vivo matured oocytes were collected from non-pregnant females. Methylation of imprinted genes was determined by restriction enzyme based assays, and transcript abundance of imprinted and nutrient supply genes were analyzed by quantitative PCR (qPCR).
Results Maternal age was associated with fetal growth restriction and placental overgrowth. In maternally aged mice, methylation was minimally dysregulated in fetal tissue, while placental tissue showed aberrant methylation and transcript abundance of imprinted genes. Ovarian methylation and gene expression was severely dysregulated, although oocyte gene expression was only minimally altered. Abundance of *Kcnq1* transcripts was significantly ($P<0.05$) increased in oocytes obtained from aged females compared to young females. Gene expression was also severely dysregulated in the uterus, including nutrient transport genes.
Conclusion Fetal and placental growth abnormalities correspond to aberrant methylation and gene expression in reproductive tissues from maternally aged mice. Significant alterations in gene expression and methylation in the aged ovary suggests that the follicular environment may be compromised. Aberrant methylation and expression of imprinted genes in the aged uterus may contribute to reduced implantation. Maternal age negatively affects imprinted gene methylation and expression in both germ cells and somatic cells of the reproductive tract, contributing to the reduced fertility observed with advanced maternal age.

Keywords Maternal age · Epigenetics · Fetal development · Methylation

Capsule Maternal age dysregulates the methylation and expression patterns of imprinted genes in reproductive tissues of mice, resulting in abnormal fetal and placental development.

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Introduction

The percentage of women postponing childbearing into their 30s and 40s has increased as a result of delayed marriage, advances in contraceptive methods, and the pursuit of higher education and careers [1]. However, delaying pregnancy has increased the reliance on assisted reproduction due to decreased natural pregnancy success with increasing maternal age [2–5]. Over 176,000 assisted reproductive technology (ART) cycles were performed in 2012; 60 % of women who underwent an assisted reproduction cycle were over the age of 35, and 17 % were over the age of 40 [2]. Unfortunately, the success of ART cycles also decreases as maternal age increases. Pregnancy success for women less than 35 years of age, transferring fresh embryos from nondonor eggs, was 47 % in 2012, while for women greater than 40 years of age, pregnancy success decreased to 15 % [2]. Advanced maternal age also affects reproductive performance in other species; aged females produce fewer oocytes in mice [6, 7], horses [8], hamsters [9], and rhesus monkeys [10], and have

decreased fertilization and embryo development in hamsters [9], mares [11], and mice [2, 12].

The role of uterine factors in the diminished reproductive efficiency of aged females is unclear. A uterine factor is not likely a significant cause for diminished pregnancy in aged women, as patient age does not have an effect on the percentage of cycles resulting in a pregnancy after transfer of fresh or frozen embryos from donor oocytes [2], suggesting that oocyte quality is the major contributing factor to diminished fertility. However, pregnancy is significantly reduced after transfer of embryos obtained from young females into uteri of aged females in both mice [12, 13] and hamsters [9]; thus, a uterine factor may also be involved in the decreased fecundity of aged females, and there could be species differences in how maternal age effects uterine receptivity.

Oocytes derived from aged mice and women display altered expression of essential genes involved in oocyte development. In humans, genes involved in cell cycle checkpoint, DNA damage response and repair, and transcription were decreased in oocytes from aged women [14]. Transcript abundance of genes involved in establishing and maintaining DNA methylation were altered in oocytes derived from aged mice [15], suggesting that genomic imprinting in oocytes obtained from aged females may be impaired. Epigenetic modifications alter gene expression without altering the DNA sequence, thereby generating a phenotypic change without modifying the genotype [16]. DNA methylation and histone acetylation are common epigenetic modifications that silence or activate gene expression, respectively, by altering the ability of transcription factors to access DNA and initiate transcription. Genomic imprinting is an epigenetic modification that generally results in parent-of-origin allele specific gene expression through inactivation of one parental allele via methylation. The intergenomic conflict hypothesis theorizes that maternal expression (paternal silencing/methylation) of imprinted genes distribute maternal nutrient resources equally among current and future offspring, while maintaining the health of the female. Paternally expressed genes (maternal silencing/methylation) promote fetal and placental growth by maximizing maternal resources to the current offspring at the expense of the maternal health and future generations [17–19]. Knockout studies of maternally expressed genes result in fetal and placental overgrowth, whereas knockout of paternally expressed genes result in fetal and placental growth restriction [17]. Dysregulation of methylation patterns may be an indicator for epigenetic instability which could result in infertility [20]. There is a potential link between maternal age and imprinting disorders, such as Angelman syndrome and Beckwith-Wiedermann syndrome, as these disorders are more prevalent in the offspring from older women [20, 21]. Furthermore, maternal age has been associated with developmental abnormalities, including low birth weight in women [3, 22, 23] and mares [24].

The objective of this experiment was to assess pregnancy outcome, fetal and placental development, and transcript abundance and methylation of imprinted genes in ovarian, uterine, fetal, and placental tissue isolated from aged (15 months) and young (4–5 weeks) mice. Our hypothesis was that dysregulation of methylation and imprinted gene expression in the ovary, uterus, placenta, and fetus is associated with the abnormal fetal and placental development associated with advanced maternal age.

Materials and methods

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

Animals

All mouse protocols followed animal care and use guidelines, as described by the *Guide for the Care and Use of Laboratory Animals* [25]. The ages of mice selected for this study (young, 4–5 weeks; aged, 15 months) were based on recent literature and breeding guidelines. Female mice, approximately 3–5 weeks of age, are developmentally competent [26] and recommended for superovulation, natural mating, and embryo recovery [27]. Mice are generally retired from breeding around 9 months of age [27]; however, females are still capable of ovulating and carrying offspring, albeit at a lower frequency than their younger counterparts. Previous reports have been successful in stimulating and collecting metaphase II oocytes from mice ranging from 10 to 16 months of age [7, 15, 28–31]. B6D2F1 mice are considered exceptional breeders, as females regularly produce large litters (JAX® Mice; Jackson Laboratory, Bar Harbor, ME) and have a long lifespan of approximately 26 months on average (Harlan Laboratories, Indianapolis, IN). According to the National Vital Statistics Reports, the estimated life expectancy for women is 80 years [32]; therefore, the mice in our experiment, aged 15 months, would roughly correspond to women in their mid-forties, assuming a linear relationship.

Tissue collection

Young (n=25) and aged (n=25) B6D2F1 mice were randomly housed with intact B6D2F1 males, regardless of the stage of estrous cycle, until a copulation plug was observed (morning of plug=d0.5). Females that displayed a copulation plug were removed and housed individually. Females that failed to display a copulation plug after 1 month were removed from the analysis (two young and three aged females were excluded). On d16.5 post copulation, females were euthanized and the number of females pregnant and the numbers of normal and degenerating implantation sites were recorded. Fetal and

placental tissues were dissected from two randomly selected littermates from three young (n=6) and three aged (n=6) females, and weighed individually. Fetal tissue was dissected longitudinally, and placentas were dissected in half. Ovarian tissue from three pregnant young and aged females was collected and extraneous tissue was removed. Right and left designation of tissues was recorded when applicable, and dissected samples were snap frozen individually in liquid nitrogen and stored at −80 °C prior to analysis.

In vivo matured oocytes and uterine tissue were collected from non-pregnant aged and young mice post superovulation with pregnant mare serum gonadotropin (PMSG, 5 IU, i.p.; Calbiochem, La Jolla, CA) followed by human chorionic gonadotropin (hCG, 5 IU, i.p.; Calbiochem) 48 h later. Uterine tissue was snap frozen in liquid nitrogen and stored at −80 °C prior to analysis. Metaphase II stage oocytes were collected 14 h post-hCG injection by oviductal dissection and denuded via pipetting in G-mops (Vitrolife, Englewood, CO) supplemented with 5 % fetal calf serum (FCS; Hyclone, Logan, UT). Oocytes that had reached metaphase II, indicated by the extrusion of the first polar body, were frozen at −80 °C in three groups of 20 oocytes per treatment in PicoPure lysis buffer (Applied Biosystems; Carlsbad, CA).

Quantitative PCR

Tissue samples were homogenized and total RNA was extracted from 30 mg of homogenized tissue using the RNeasy Mini Kit and on-column DNase treatment (RNase-Free DNase Kit; Qiagen; Valencia, CA). Fetal and placental tissues obtained from two littermates per female were homogenized and analyzed individually (n=6 samples per treatment per tissue). RNA was quantified using the Quant-iT RiboGreen RNA Assay Kit (Invitrogen; Carlsbad, CA) and single strand cDNA were generated by random primed cDNA synthesis from 5 μg of RNA. cDNA samples were diluted 25-fold for a final concentration of 20 ng/μL.

Total RNA from oocytes was extracted using the PicoPure RNA Isolation Kit (Applied Biosystems) followed by an on-column DNase treatment (RNase-Free DNase Kit; Qiagen). cDNA was generated using the Sensiscript Reverse Transcription Kit (Qiagen), following the manufacturer's protocol with a 3 h incubation period.

Imprinted genes previously associated with fetal and placental growth dysregulation, as well as genes involved in nutrient supply, were analyzed by quantitative PCR (qPCR). Genes analyzed in this study included maternally expressed genes (cyclin-dependent kinase inhibitor 1C, *Cdkn1c*; guanine nucleotide binding protein, alpha stimulating, *Gnas*; growth factor receptor bound protein 10, *Grb10*; gene-trap locus 2, *Gtl2*; H19, imprinted maternally expressed transcript, *H19*; insulin-like growth factor 2 receptor, *Igf2r*; potassium voltage-gated channel, subfamily Q, member 1, *Kcnq1*; achaete-scute complex homology 2, *Mash2*; pleckstrin homology-like domain, family 2, *Phlda2*), paternally expressed genes (insulin-like growth factor 2, *Igf2*; mesoderm specific transcript, *Mest*; paternally expressed 3, *Peg3*; small nuclear ribonucleoprotein N, *Snrpn*), or genes involved in nutrient supply (lipoprotein lipase, *Lpl*; solute carrier family 2, member 3, *Slc2a3*; solute carrier family 38, member 4, *Slc38a4*). A summary of the target and reference genes, including accession number, primer sequence, and product length, is presented in Table 1. Primer design and qPCR were completed as previously described [33, 34]. Briefly, primers were designed for qPCR using Primer3 [35] and primer specificity was determined by melt curve analysis and gel electrophoresis. PCR products were cloned into pCR 2.1 TOPO vectors and transformed into One Shot TOP10 chemically competent *E. coli* (Invitrogen). Plasmids were sequenced to confirm the identity of the transcript and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Quantitative PCR assays for ovarian, uterine, fetal, and placental tissue were performed with 100 ng cDNA in a 15 μL reaction of iQ SYBR Green Supermix (Bio-Rad; Hercules, CA) and analyzed in duplicate. Quantitative PCR for oocytes was performed on 10-fold diluted cDNA in a 15 μL reaction run in duplicate using QuantiFast SYBR Green Supermix (Qiagen). A standard curve was generated from serial dilutions of EcoRI digested plasmids (10^7 to 10^1 molecules) to calculate log starting quantity.

Methylation analysis

Ovarian and uterine tissues were collected and frozen as described above, with three females represented per treatment. Fetal and placental tissue from one or two littermates from each of the three females per treatment were collected and frozen as described above (n=4 fetal and placental samples per treatment per tissue). When possible, tissue from the same mice used to determine transcript abundance was also used for methylation analysis (n=24 out of 28). Approximately 20 mg of tissue was homogenized and DNA was isolated using the QIAamp DNA Mini Kit (Qiagen). DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) and 1.5 μg of DNA was used to determine percent methylation. Genes analyzed for methylation were *Cdkn1c*, *Grb10*, *Gnas*, *Igf2*, *Igf2r*, *Kcnq1*, *Mest*, and *Peg3*; accession number and CpG island location are presented in Table 2. Two CpG islands for *Gnas* and *Grb10* were analyzed and designated as either (1) or (2).

Methylation status of ovarian, uterine, fetal and placental tissues was analyzed using the EpiTect Methyl II Custom PCR Array (Qiagen), with the genes of interest pre-loaded onto the array, following the manufacturer's protocol, with slight modifications. Briefly, 1.5 μg of extracted genomic DNA was incubated with 5× Restriction Digestion Buffer and equal

Table 1 Summary of quantitative PCR primers for target and reference genes

Gene symbol	Accession number	Forward primer sequences	Reverse primer sequence	Product length
Cdkn1c	NM_009876	5'-gaaccgctgggacttcaa-3'	5'-gtagaaggcgggcacaga-3'	100 bp
Gnas	NM_022000	5'-tccctgagtcctctgaatctg-3'	5'-tctggctcggtctcgatt-3'	172 bp
Grb10	NM_010345	5'-cgacactggaacggagaag-3'	5'-agagggaggtggtcctgttt-3'	172 bp
Gtl2	NR_003633	5'-ggagacccgctctgagtta-3'	5'-gtcaggacagggagttgtga-3'	172 bp
H2afz	NM_016750	5'-acagcgcagccatcctggagta-3'	5'-ttcccgatcagcgatttgtgga-3'	202 bp
H19	X58196	5'-ccacccaccgtaattcactt-3'	5'-aaatccctctggagtccgata-3'	159 bp
Igf2	NM_010514	5'-gagttcagagaggccaaacg-3'	5'-tagtgtgggacgtgatggaa-3'	189 bp
Igf2r	NM_010515	5'-aggcatggcaatttgtatga-3'	5'-tttcctggcatgatgagact-3'	157 bp
Kcnq1	NM_008434	5'-gcaacagcaggtcacaagtt-3'	5'-ttaccctggacctcccttct-3'	162 bp
Lpl	NM_008509	5'-ttcattgactccctgctgaa-3'	5'-gcttctcttggctctgacct-3'	153 bp
Mash2	NM_008554	5'-gactcctggtggacctacct-3'	5'-gcagaggtcagtcagcactt-3'	107 bp
Mest	NM_008590	5'-ttcatcggctgcttctaaca-3'	5'-ctgaacccaagggctttaac-3'	114 bp
Peg3	NM_008817	5'-tatgaagggagcgcatttag-3'	5'-tcatctcagcaccacactca-3'	172 bp
Phlda2	NM_009434	5'-ttccactccatcctcaaggt-3'	5'-cggttctggaagtcgatca-3'	158 bp
Slc2a3	NM_011401	5'-cttatgggattcgccaagat-3'	5'-tggtttagtgtgccaaatgc-3'	158 bp
Slc38a4	NM_027052	5'-cggaaatctgacgttcaaca-3'	5'-acgccattgctgtgaagag-3'	159 bp
Snrpn	NM_013670	5'-tcacttgtacccacgacgtt-3'	5'-aaccacctcttggtgtctca-3'	101 bp

amounts of DNA were loaded into four reactions: a mock reaction (M_o), containing no restriction digestion enzyme; a methylation-sensitive enzymatic reaction (M_s), which detects methylated DNA sequences; a methylation-dependent enzymatic reaction (M_d), which detects unmethylated DNA sequences; and a double enzymatic reaction (M_{sd}), containing both methylation-sensitive and methylation-dependent restriction digestion enzymes, which digests both methylated and unmethylated DNA and measures the background of the assay (EpiTect II DNA Methylation Enzyme Kit; Qiagen). Samples were incubated overnight at 37 °C followed by 20 min at 65 °C to deactivate the restriction digest enzymes. Quantitative PCR was performed on the digested samples with RT^2 SYBR Green ROX qPCR Mastermix (Qiagen) using an Applied Biosystems 7900 HT Fast Real Time PCR System with the following thermal cycler protocol: 1) 95 °C for 10 min; 2) 3 cycles at 99 °C for 30 s followed by 72 °C for 15 s; 3) 40 cycles at 97 °C for 15 s followed by 72 °C for 1 min; and 4) melt curve analysis according to instrument recommendations. Methylation-sensitive enzyme (catalog number EPHS115450-1A; SEC) and methylation-dependent enzyme control primers (catalog number EPHS115451-1A; DEC) were also analyzed for each tissue to monitor the efficiency of enzymatic digestion. For data analysis, the raw threshold cycle (C_T) data was exported into the EpiTect Methyl II PCR Array Microsoft Excel based data analysis template (Qiagen) and percent methylation was calculated following the manufacturer's protocol.

Imprinted genes were selected for analysis based on current literature demonstrating abnormal effects of altered methylation or expression on fetal and placental development [17, 19, 36, 37]. As the methylation assays used in the current experiment are based on restriction enzyme digestion, a specific

Table 2 Summary of primers to determine percent methylation in the mouse

Gene symbol	Catalog number	Accession number	CpG island location
Cdkn1c	EPMM110574-1A	NM_009876	Chr7: 150644960–150647382
Gnas (*1*)	EPMM106893-1A	NM_201616	Chr2: 174152794–174156646
Gnas (*2*)	EPMM106888-1A	NM_022000	Chr2: 174110148–174110891
Grb10 (*1*)	EPMM101357-1A	NM_001177629	Chr11: 11925557–11926335
Grb10 (*2*)	EPMM101358-1A	NM_010345	Chr11: 11936492–11938111
Igf2	EPMM110567-1A	NR_002855	Chr7: 149844853–149847223
Igf2r	EPMM104610-1A	NM_010515	Chr17: 12961924–12962875
Kcnq1	EPMM110573-1A	NM_008434	Chr7: 150292933–150293670
Mest	EPMM109182-1A	NM_008590	Chr6: 30687907–30688499
Peg3	EPMM109727-1A	NM_008817	Chr7: 6682773–6683109

level of methylation is required for the enzyme to effectively cut the DNA; therefore, the availability of genes for methylation analysis was limited by the ability to obtain primers within known CpG islands from the manufacturer. It is also important to note, with the utilization of the EpiTect Methyl II Custom PCR Arrays, methylation analysis in the current experiment looked at only one or two specific CpG islands per gene which covers a relatively small portion of the complete gene of interest. Therefore, the methylation analysis presented in this study represents only the percent methylation at specific CpG islands and may not reflect total methylation status when looking at the entire gene. We did not determine percent methylation of imprinted genes in oocytes due to lack of availability of maternally aged oocytes in the numbers required for the EpiTect Methyl II Custom PCR Array.

Statistical Analysis

Fetal and placental weights, number of normal and degenerating implantation sites, and pregnancy outcome were analyzed using the general linear model of ANOVA with female mouse ID nested within age as a random effect, and age as the main fixed factor. For the fetal and placental analyses, the unit of comparison was the pup nested within the litter to account for any differences due to litter/dam. Log values for transcript abundance for each sample were analyzed in duplicate using the non-parametric Mann–Whitney U *t*-test. Percent methylation was analyzed using a Chi-Square Test. For all analyses, statistical difference was defined with a P-value less than 0.05.

For relative quantification of qPCR data for young and aged oocytes, the threshold for each target gene was adjusted to the threshold level of the control gene (*H2afz*). H2A histone family, member Z (*H2afz*) was selected as a control gene as transcript abundance remained stable (not statistically different) between treatment groups. Data were analyzed using the relative expression software tool, REST 2005 version 1.9.12 [38], with *H2afz* set as the reference gene. Expression ratios were generated from equation 1 using PCR efficiencies (E) of the target and reference genes and the ΔCT values of the control (young) and sample (aged) oocytes. The threshold cycle (CT) values represent the PCR cycle when the SYBR Green fluorescence rises above the background fluorescence, or threshold. The levels of significance were calculated by pair-wise fixed reallocation randomization tests with 50,000 iterations and significance was determined with a P-value less than 0.05.

$$ratio = \frac{(E_{target})\,\Delta CT\,target\,(control - sample)}{(E_{ref})\,\Delta CT\,ref\,(control - sample)} \quad (1)$$

Results

Fetal and placental development

Pregnancy success and fetal and placental development of young (4–5 weeks) and aged (15 months) mice were assessed on d16.5 post copulation after natural mating. A higher percentage of young female mice were pregnant compared to aged females (Table 3; $P<0.05$). On average, time to pregnancy was shorter in aged (4.47±0.92 days) compared to younger females (8.09±0.77 days; $P<0.05$). Young mice had a higher number of normal implantation sites compared to aged mice and a significantly reduced number of degenerating implantation sites compared to aged mice (Table 3; $P<0.05$). Average weight of d16.5 fetuses derived from young females was significantly higher compared to fetuses derived from aged females (Table 3; $P<0.05$); however, the average weight of placentas derived from younger females was significantly lower compared to placentas from aged females (Table 3; $P<0.05$).

Quantitative PCR

Transcript abundance of 13 imprinted genes was analyzed by qPCR in dissected fetal, placental, uterine, and ovarian tissue harvested from young and aged mice. Transcript abundance was not significantly different in fetal tissue from aged mice compared to fetuses from young mice ($P>0.05$). Transcript abundance of three maternally (*Cdkn1c*, *Grb10*, and *Kcnq1*) and one paternally (*Peg3*) expressed gene were significantly lower in placentas from aged female mice ($P<0.05$; Fig. 1). In the pregnant female, expression of four paternally expressed

Table 3 Pregnancy success of young (3–4 weeks) and aged (15 months) female mice after natural mating to males, indicated by percentage pregnant at d16.5 post copulation, average number of normal and degenerating implantation sites, and average fetal and placental weights

Age	Percent pregnant (M±SE)[1]	No. normal sites per pregnant female (M±SE)	No. degenerating sites per pregnant female (M±SE)	No. of fetuses	Fetal weight (M±SE)	No. of placentas[2]	Placental weight (M±SE)
Young	65.22±10.15 % (15/23)[a]	7.87±0.27[a]	0.07±0.07[a]	118	0.47±0.01g[a]	114	0.08±0.01g[a]
Aged	27.27±9.72 % (6/22)[b]	4.00±0.37[b]	1.50±0.43[b]	24	0.29±0.01g[b]	19	0.11±0.01g[b]

[ab] Different superscripts within a column denote significant difference, $P<0.05$

[1] Percentage of females pregnant d16.5 after a coagulation plug was observed

[2] Extraembryonic membranes were removed and non-intact placentas were excluded from the analysis

genes (*Igf2*, *Mest*, *Peg3*, and *Snrpn*) and eight maternally expressed genes (*Cdkn1c*, *Gnas*, *Grb10*, *Gtl2*, *H19*, *Igf2r*, *Kcnq1*, and *Phlda2*) were elevated in ovarian tissue from aged mice ($P<0.05$; Fig. 2). Transcript abundance of three paternally expressed (*Igf2*, *Mest*, and *Peg3*) and nine maternally expressed genes were significantly decreased in uterine tissue obtained from non-pregnant aged females compared to young females ($P<0.05$; Fig. 3). Abundance of three genes involved in nutrient transport, *Slc2a3*, *Slc38a4*, and *Lpl*, was also investigated in fetal, placental, ovarian and uterine tissue. All three genes were not significantly different in fetal and placental tissue derived from aged and young mice ($P>0.05$). In ovarian tissue from pregnant females, all three genes were up regulated in aged females, whereas in uterine tissue, *Lpl* and *Slc38a4* were down regulated in non-pregnant aged females compared to young females ($P<0.05$; Figs. 2 and 3).

Transcript abundance was analyzed by qPCR in in vivo matured metaphase II oocytes harvested from young and aged mice. Abundance of *Kcnq1* was eight-fold higher ($P<0.05$) in oocytes obtained from aged females compared to young females. *Igf2* was not detectable in oocytes from either aged or young females. The 14 remaining genes were present but were not significantly different between oocytes obtained from young or aged females ($P>0.05$).

Methylation

Percent methylation remained relatively stable for most genes in fetal tissues derived from young and aged mice ($P>0.05$). Methylation of the *Grb10*(*2*) (13.06 %±12.71 % vs. 0.08 %± 0.03 %) and *Kcnq1* (13.80 %±12.91 % vs. 0.23 %±0.10 %) CpG islands in fetal tissue were significantly increased ($P<0.05$) in aged females compared to young females, respectively; however, methylation of the *Cdkn1c* CpG island was significantly decreased in aged mice (36.58 %±18.98 %) compared to young mice (59.44 %±0.59 %; $P<0.05$). Placental tissue from aged mice had significantly ($P<0.05$) increased methylation levels for all genes analyzed, except for *Grb10*(*2*) which was not significantly different compared to young females ($P>0.05$; Fig. 4). Ovarian tissue from pregnant aged mice had significantly ($P<0.05$) decreased methylation levels for all genes analyzed, except for *Grb10*(*1*) and *Grb10*(*2*), which were not significantly different compared to pregnant young females ($P>0.05$; Fig. 5). Percent methylation of uterine tissue was significantly lower for *Grb10*(*2*) in non-pregnant aged mice compared to non-pregnant young mice; however, percent methylation of *Igf2* and *Mest* were significantly higher in uterine tissue obtained from aged mice compared to younger mice, respectively ($P<0.05$; Fig. 6). Methylation of *Peg3* was not detectable in uterine tissues derived from aged mice.

Discussion

In this experiment, we demonstrate alterations in fetal and placental development, as well as corresponding alterations in both methylation and transcript abundance of paternally and maternally expressed genes in germ cells and somatic cells of reproductive tissues from aged females. Based on the intergenomic conflict hypothesis, the severe fetal growth restriction and overgrowth of the placenta from maternally aged mothers that we observed indicates that the balance between paternally and maternally imprinted genes is altered, which agrees with our results that methylation status and gene expression is indeed different in aged compared to young mice. Oocyte yield post superovulation for B6D2F1 young and aged mice has been previously reported [30]; oocyte

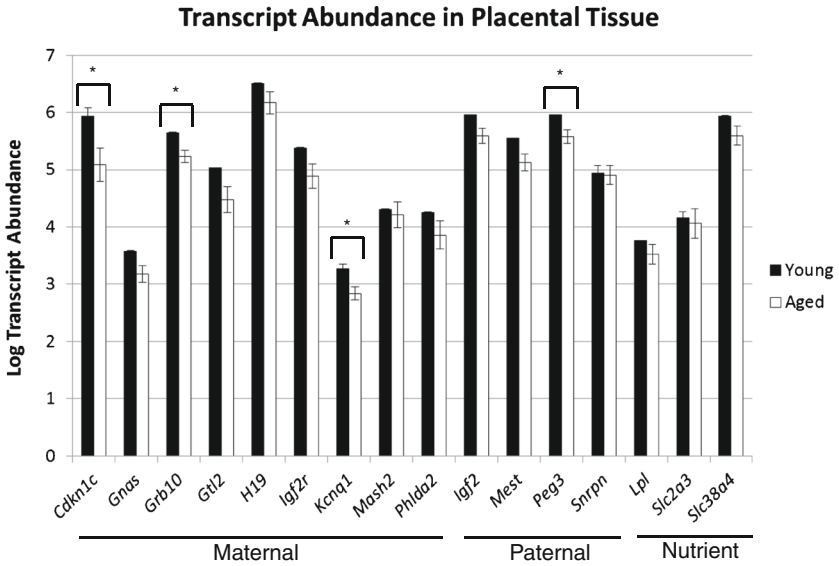

Fig. 1 Transcript abundance in placental tissue derived from young (4–5 weeks; *black bars*) and aged (15 months; *white bars*) mice. Genes analyzed were maternally expressed (*Cdkn1c*, *Gnas*, *Grb10*, *Gtl2*, *H19*, *Igf2r*, *Kcnq1*, *Mash2*, and *Phlda2*), paternally expressed (*Igf2*, *Mest*, *Peg3*, and *Snrpn*), or involved in nutrient supply (*Lpl*, *Slc2a3*, and *Slc38a4*). *, $P<0.05$

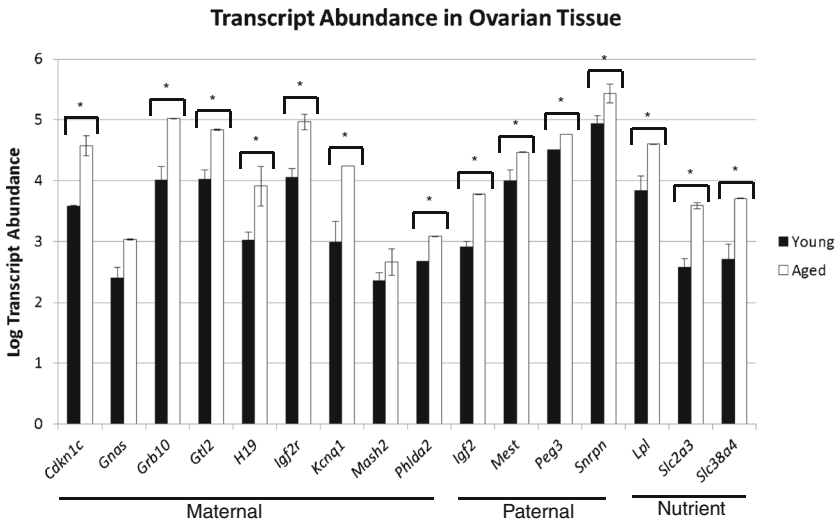

Fig. 2 Transcript abundance in ovarian tissue derived from pregnant young (4–5 weeks; *black bars*) and aged (15 months; *white bars*) mice. Genes analyzed were maternally expressed (*Cdkn1c*, *Gnas*, *Grb10*, *Gtl2*, *H19*, *Igf2r*, *Kcnq1*, *Mash2*, and *Phlda2*), paternally expressed (*Igf2*, *Mest*, *Peg3*, and *Snrpn*), or involved in nutrient supply (*Lpl*, *Slc2a3*, and *Slc38a4*).*, $P<0.05$

recovery from mice 6–8 weeks old was 39.5±7.4 oocytes compared to 4.4±3.6 oocytes from mice aged 16 months [30]. Fetal growth restriction is also associated with advanced maternal age in women [3, 22, 23] and mares [24], and there is a higher incidence of embryonic loss and miscarriage in older women [2–4, 12] and mares [8, 11], consistent with the increased number of degenerating implantation sites in aged mice observed in this experiment. Also in agreement with our findings, maternal age in women is associated with abnormal placental growth; women of advanced maternal age have increased placental weights compared to younger women [39].

Aberrant methylation patterns of three maternally expressed imprinted genes (*Cdkn1c*, *Grb10*, and *Kcnq1*) were observed in fetal tissues obtained from aged mice. A two-fold increase in *Cdkn1c* transcript abundance can cause a 10–30 % reduction in fetal growth [40]. The decrease in *Cdkn1c* methylation we observed in the current experiment should result in overexpression of the gene and would thus contribute to the fetal growth restriction in aged mice that we observed; however, aberrant gene expression of *Cdkn1c* was not detected. In contrast, methylation of *Grb10* and *Kcnq1* were increased in fetal tissue from aged mothers, suggesting a decrease in gene expression that should correspond to an increase in fetal weight according to the intergenomic conflict hypothesis [19]; however, this is inconsistent with the fetal development we observed and with our gene expression analysis that showed no difference in transcript abundance for *Grb10* or *Kcnq1*. The lack of differences in transcript abundance in the fetal tissue could be due to the presence of numerous tissue types within the fetal sample, as individual tissues within the fetus were not dissected prior to analysis. Dissecting individual organs from the fetuses may provide information on the individual cell or tissue type but not on the global gene expression or methylation patterns in the reproductive tissue. While the fetal tissue did not show significant differences in

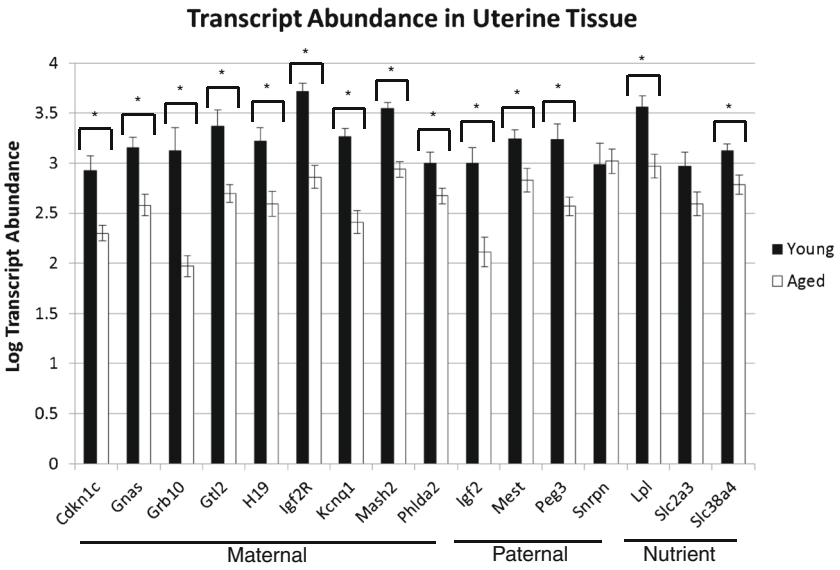

Fig. 3 Transcript abundance in uterine tissue derived from non-pregnant young (4–5 weeks; *black bars*) and aged (15 months; *white bars*) mice. Genes analyzed were maternally expressed (*Cdkn1c*, *Gnas*, *Grb10*, *Gtl2*, *H19*, *Igf2r*, *Kcnq1*, *Mash2*, and *Phlda2*), paternally expressed (*Igf2*, *Mest*, *Peg3*, and *Snrpn*), or involved in nutrient supply (*Lpl*, *Slc2a3*, and *Slc38a4*).*, $P<0.05$

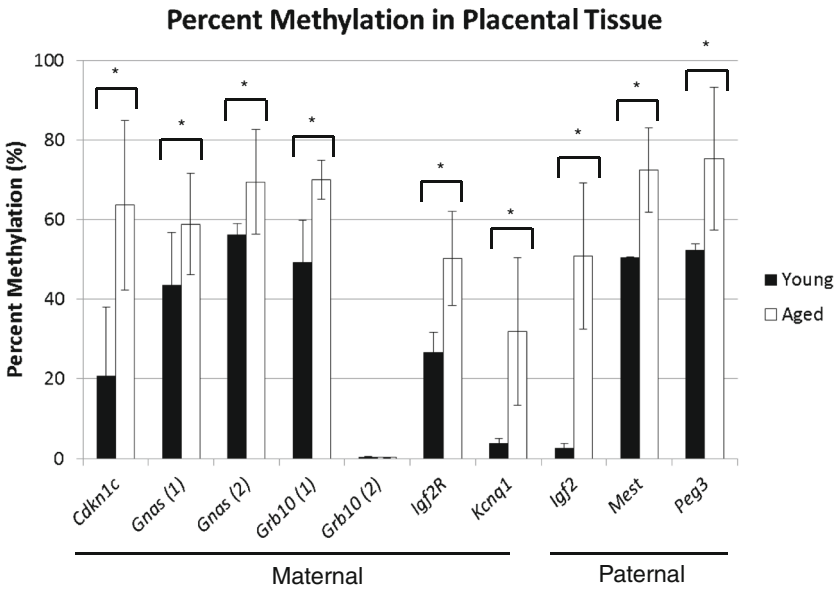

Fig. 4 Percent methylation of target genes in placental tissue derived from young (4–5 weeks; *black bars*) and aged (15 months; white bars) mice. CpG islands located on maternally expressed (*Cdkn1c*, *Gnas*, *Grb10*, *Igf2r*, and *Kcnq1*) and paternally expressed (*Igf2*, *Mest*, and *Peg3*) genes were analyzed.*, $P<0.05$

gene expression which may be in part due to the numerous tissue types present within the fetus, we did see significant changes in methylation patterns which provides valuable information on the function of the tissue as a whole. Alternatively, other post-transcriptional mechanisms controlling transcript stability and degradation may also influence the levels of transcripts that we observed.

Our findings support the concept that not all imprinted genes follow the intergenomic conflict hypothesis. Differential expression of maternally expressed genes was also reported in intrauterine growth restricted associated human placentas. An increase in abundance of *Phlda2* and *Cdkn1c*, two maternally expressed genes, were observed in intrauterine growth restricted placentas, which is in agreement with the intergenomic conflict hypothesis [41]. However, intrauterine growth restriction also coincided with a decrease in *Gnas*, *Meg3*, and *Gatm*, which are also maternally expressed, and therefore opposes the intergenomic conflict hypothesis [41]. Furthermore, abundance of *Peg10* and *Nnat*, two paternally expressed genes, are elevated in intrauterine growth restricted placentas, which also opposes the intergenomic conflict hypothesis [42]. One theory proposed to explain why genes sometimes do not follow the intergenomic conflict hypothesis suggests that the copy number of an imprinted gene may be more important in regulating aberrant development rather than altered imprinting status. A male patient with duplication of the paternal 7p12.2, corresponding to the maternally expressed gene *Grb10*, displayed increased height and weight rather than the growth restriction expected with increased *Grb10* expression [43]. However, a female patient with

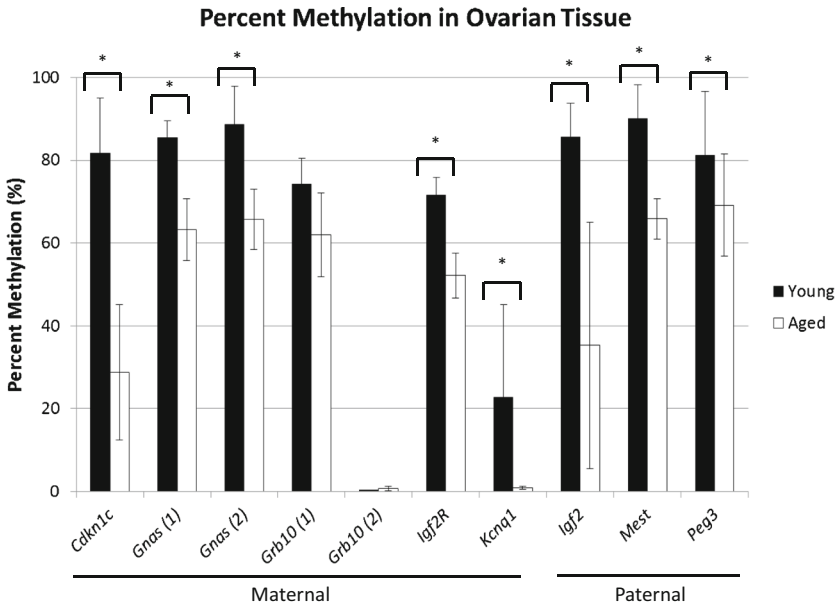

Fig. 5 Percent methylation of target genes in ovarian tissue derived from pregnant young (4–5 weeks; *black bars*) and aged (15 months; *white bars*) mice. CpG islands located on maternally expressed (*Cdkn1c*, *Gnas*, *Grb10*, *Igf2r*, and *Kcnq1*) and paternally expressed (*Igf2*, *Mest*, and *Peg3*) genes were analyzed.*, $P<0.05$

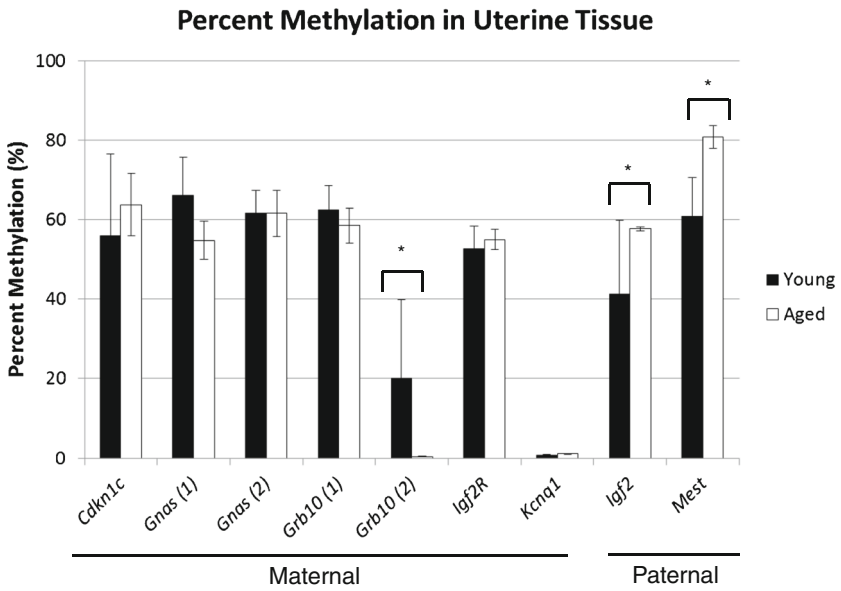

Fig. 6 Percent methylation of target genes in uterine tissue derived from non-pregnant young (4–5 weeks; *black bars*) and aged (15 months; *white bars*) mice. CpG islands located on maternally expressed (*Cdkn1c*, *Gnas*, *Grb10*, *Igf2r*, and *Kcnq1*) and paternally expressed (*Igf2* and *Mest*) genes were analyzed.*, $P<0.05$

paternal duplication in the same region displayed features of Silver-Russell Syndrome, including decreased birth weight and stature [44], suggesting that the mechanisms that regulate aberrant development are still largely unknown.

Transcript abundance of four maternal (*Cdkn1c*, *Gnas*, *Grb10*, and *Kcnq1*) and two paternal genes (*Peg3* and *Mest*) were decreased in placental tissue obtained from aged mice. In agreement with the decreased transcript abundance of imprinted genes, methylation for *Cdkn1c*, *Gnas*, *Grb10*, *Igf2*, *Igf2r*, *Kcnq1*, *Mest* and *Peg3*, were increased in placental tissue derived from aged mice. The increased methylation and decreased transcript abundance of maternally expressed genes, and the corresponding placental overgrowth observed in the current experiment, supports the intergenomic conflict hypothesis [17–19]. Interestingly, we also observed a decrease in gene expression of several paternally expressed genes which, according to the intergenomic conflict hypothesis, should result in placental growth restriction. These findings further support the theory that not all imprinted genes follow the intergenomic conflict hypothesis [41–43], similar to what we observed in fetal tissue.

Uterine tissue harvested from non-pregnant aged mice showed minor changes in methylation but severe dysregulation in transcript abundance. The uterine environment is essential for embryo implantation and development of the fetus; alterations in transcript abundance in aged females may indicate a decreased ability of the uterus to support implantation and/or the needs of the developing fetus, further compounding the decreased ability of aged mice to produce offspring. Changes in uterine gene expression may indicate inadequacy of the endometrium, including decreased uterine receptivity [12, 45], delayed secretory maturation of the endometrium [46], and uterine fibroids [47], which have been observed in aged women. The uterine tissue used in the current study was not dissected to separate the endometrium, myometrium or perimetrium; therefore, we cannot conclude that the effects of maternal age are solely due to alterations in the endometrium. Even though the non-pregnant uterus was evaluated in this experiment, our results suggest that the uterine environment in aged females may not be as conducive to developing a receptive endometrium and establishing a pregnancy compared to young females.

Supply and demand of maternal nutrient resources have a significant impact on fetal and placental development. Decreased nutrient supply has been associated with fetal growth restriction and smaller placental weights in guinea-pigs [48, 49], sheep [50], rats [51], and cattle [52]. Growth restricted placentas in the mouse are capable of maintaining normal fetal growth until late in gestation, in part by increasing the expression of glucose (*Slc2a3*/GLUT3) and System A amino acid (*Slc38a4*/SNAT4) transporters [53]. In the current study, reduced fetal weight in aged mice may indicate that nutrient supply and transport to the fetus are reduced. Interestingly, we did not see a difference in the abundance of *Scl2a1*, *Slc38a4*, or *Lpl* in the placental tissue between young and aged mice. The placental overgrowth observed in aged females suggests compensation for poor nutrient delivery by increasing the size of the placenta to support fetal development, rather than up regulation of nutrient transporters.

Both maternally and paternally expressed genes displayed a significant decrease in methylation in ovarian tissue obtained from pregnant aged females compared to younger females, which corresponded to a significant increase in transcript abundance in aged mice. This may indicate that these dysregulated genes result in compromised follicle development, oocyte growth, and oocyte quality, leading to perturbed embryonic and fetal development. However, only one imprinted gene, *Kcnq1*, displayed altered transcript abundance in

in vivo matured oocytes; therefore, the dysregulation in methylation and gene expression observed in the ovary may be isolated to stroma, granulosa, theca or cumulus cells, potentially interfering with follicular recruitment, environment and/or cumulus cell-oocyte communication. It is well known that somatic cell-oocyte communication is essential to support oocyte development competence [54]. Thus, altered methylation and transcript abundance in ovarian somatic cells may have an indirect impact on oocyte development and quality, contributing to the observed developmental anomalies in fetal and placental development in the offspring from aged mice.

In summary, maternally aged mice exhibited fetal growth restriction, placental overgrowth, and disruption in the balance between maternally and paternally expressed genes due to alterations in methylation regulating gene expression. Concurrent up and down regulation of both maternally and paternally expressed genes in reproductive tissues derived from aged mice supports the concept that not all imprinted genes follow the intergenomic conflict hypothesis. Even though maternally aged women have an equal chance of pregnancy after ART cycles using donor oocytes, data from the current experiment suggests that the uterine environment may also be compromised in mice. While minimal alterations were observed in expression of imprinted genes in the oocyte, aberrant methylation patterns and gene expression were observed in ovarian tissue from aged mice. Function of the supporting somatic cells of the ovary, and potentially the follicular environment, may thus be compromised, indirectly impairing oocyte developmental competence. In conclusion, advanced maternal age in mice alters methylation patterns of imprinted genes in essential reproductive tissues, resulting in dysregulated gene expression that is associated with poor reproductive outcomes.

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