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Modulation of imprinted gene expression following superovulation

Amanda L. Fortier [a,1], Serge McGraw [a,b,1], Flavia L. Lopes [a,b], Kirsten M. Niles [a], Mylène Landry [b], Jacquetta M. Trasler [a,b,c,*]

[a] Research Institute at the Montreal Children's Hospital of the McGill University Health Centre and Department of Human Genetics, McGill University, Montreal, Quebec H3H 1P3, Canada
[b] Research Institute at the Montreal Children's Hospital of the McGill University Health Centre and Department of Pediatrics, McGill University, Montreal, Quebec H3H 1P3, Canada
[c] Research Institute at the Montreal Children's Hospital of the McGill University Health Centre and Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec H3H 1P3, Canada

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ABSTRACT

Although assisted reproductive technologies increase the risk of low birth weight and genomic imprinting disorders, the precise underlying causes remain unclear. Using a mouse model, we previously showed that superovulation alters the expression of imprinted genes in the placenta at 9.5 days (E9.5) of gestation. Here, we investigate whether effects of superovulation on genomic imprinting persisted at later stages of development and assess the surviving fetuses for growth and morphological abnormalities. Superovulation, followed by embryo transfer at E3.5, as compared to spontaneous ovulation (controls), resulted in embryos of normal size and weight at 14.5 and 18.5 days of gestation. The normal monoallelic expression of the imprinted genes *H19*, *Snrpn* and *Kcnq1ot1* was unaffected in either the placentae or the embryos from the superovulated females at E14.5 or E18.5. However, for the paternally expressed imprinted gene *Igf2*, superovulation generated placentae with reduced production of the mature protein at E9.5 and significantly more variable mRNA levels at E14.5. We propose that superovulation results in the ovulation of abnormal oocytes with altered expression of imprinted genes, but that the coregulated genes of the imprinted gene network result in modulated expression.

1. Introduction

The regulation of embryonic growth is complex and not well understood; however, embryo manipulation has been shown to result in growth defects in many species. Babies born following assisted reproductive technologies (ART) are at an increased risk of being small for gestational age (Helmerhorst et al., 2004; Jackson et al., 2004; McDonald et al., 2005).

Following superovulation, murine embryos exhibit growth restriction at mid- to late-gestation (Ertzeid and Storeng, 2001; Evans et al., 1981; Van der Auwera and D'Hooghe, 2001). One particular class of genes, the imprinted genes, are implicated in embryonic growth and development, as well as placental development (Fowden et al., 2006; Isles and Holland, 2005; Smith et al., 2006). Imprinted genes are expressed from a single allele as the result of a parent-of-origin specific pattern established during germ cell development. As imprinted genes play a key role in regulating fetal growth, we previously examined the expression of a number of imprinted genes following superovulation in the embryo and placenta at mid-gestation (Fortier et al., 2008). Superovulation followed by embryo transfer resulted in aberrant biallelic expression of *H19* and increased levels of *Igf2* mRNA in the placenta at E9.5. Interestingly, expression of *Igf2* remained monoallelic, and methylation at the *H19* differentially methylated region (DMR) was unaffected following superovulation, suggesting that DNA methylation and genomic imprinting were not the main effectors. This is in contrast to studies examining the effects of superovulation during preimplantation stages (Market-Velker et al., 2010) and to studies examining the effects of embryo culture (Mann et al., 2004; Rivera et al., 2008) which have described effects on DNA methylation following embryo manipulations. We also did not observe an effect of superovulation on the growth of embryos at E9.5. Growth restriction following superovulation and embryo transfer has been observed at later stages of gestation (E14.5 or E18.5) (Ertzeid and Storeng, 2001; Van der Auwera and D'Hooghe, 2001), however, effects on genomic imprinting have not previously been assessed at these stages. Additionally, the effects on imprinted genes observed in our previous study were confined to the placenta, which does not become critical

* Corresponding author. Address: McGill University Montreal Children's Hospital Research Institute, 4060 Ste. Catherine Street West, Montreal, Quebec H3H 1P3, Canada. Tel. : +1 (514) 412 4400x25235; fax: +1 (514) 412 4331.
E-mail address: jacquetta.trasler@mcgill.ca (J.M. Trasler).
[1] These authors contributed equally to this work.

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for the support of the embryo until about E10.5 (Anson-Cartwright et al., 2000; Guillemot et al., 1995; Ono et al., 2006; Voss et al., 2000). This suggests that aberrant expression of imprinted genes in the placenta may contribute to fetal growth restriction later in development.

In this study, we examined the effects of superovulation at later time points in gestation to determine whether effects on imprinted genes persisted and to examine growth and morphological effects on the surviving embryos. Low dose superovulation protocols were used and embryo transfer was performed to control litter size and the uterine environment. We examined the expression of a number of imprinted genes, and demonstrated that allelic expression was not affected after midgestation. Fetuses from the superovulated females were of similar weights and sizes as compared to those from the naturally cycling controls. As was seen at E9.5, we observed an increase in placental *Igf2* expression at E14.5, which was highly variable between samples. We propose that superovulation leads to the ovulation of oocytes that are impaired in their ability to regulate *Igf2* expression in the placenta during early development, and that *H19* expression is altered in response to the dysregulation of *Igf2*, in an attempt to normalize growth factor levels.

2. Materials and methods

2.1. Sample generation and animal care

All animal experiments were performed in compliance with guidelines established by the Canadian Council for Animal Care. Adult (6–8 week old) CD1 female mice (Charles River Canada, St Constant, QC) were superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin (PMSG) (Sigma–Aldrich), followed approximately 46 h later by IP injection of 5 IU human chorionic gonadotrophin (hCG) (Sigma–Aldrich). Superovulated or naturally cycling CD1 females were mated to C57Bl/6J (CAST7) male mice (Mann et al., 2003), and the presence of a copulatory plug was designated as embryonic day (E)0.5 dpc. The presence of multiple single nucleotide polymorphisms between the CD1 and C57Bl/6J (CAST7) strains allows for the differentiation of parental alleles in the allele specific expression assays described below. Blastocysts were collected from the uterine horns at 3.5 dpc into HEPES-buffered KSOM, and immediately transferred to E2.5 pseudopregnant recipient females, as previously described (Nagy et al., 2002). Each recipient received embryos from a single control or superovulated female. Average numbers (±SEM) of blastocysts transferred per female for each group, equally distributed into each uterine horn, were: Control E14.5, 7.6 ± 1.3; Superovulated E14.5, 10.5 ± 2.0; Control E18.5, 7.2 ± 0.5; Superovulated E18.5, 14.5 ± 1.5. Embryos, placentae and yolk sacs were collected at E14.5 or E18.5, relative to the recipient female. The success rates in terms of numbers of embryos collected/number of blastocysts transferred was similar for the control and superovulated groups: Control E14.5, 50%; Superovulated E14.5, 59%; Control E18.5, 50%; Superovulated E18.5, 45%. Viable embryos were weighed and examined for gross morphological defects and placentae were weighed. For the data shown in Fig. 1, embryo weights and sizes and placentae weights were averaged for all litters with three and more viable embryos. Implantation rate was calculated by dividing the sum of the viable embryos, dead embryos (those embryos lacking a heart beat) and resorption sites by the number of embryos transferred. This may underestimate the actual implantation rate if there was early postimplantation loss that we were unable to detect. Embryos without a heartbeat were not included in the resorption rate calculations; only implantation sites where embryo degeneration had begun were included. Resorption sites ranged from recent embryo death (embryo and/or placental tissue still distinguishable but degeneration has begun) to near complete clearance of detritus in the uterine horn. Samples were snap frozen in liquid nitrogen and stored at −80 °C until analyzed.

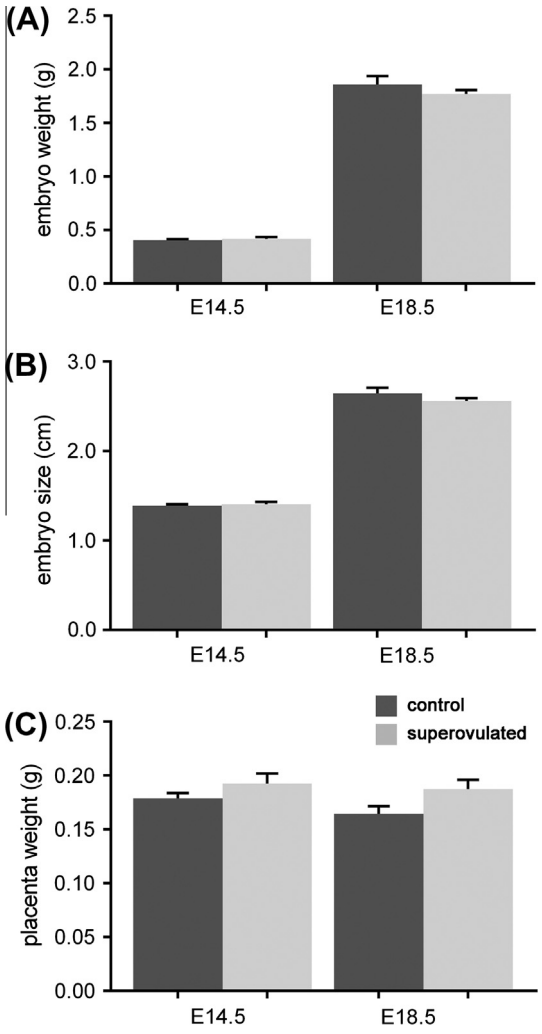

Fig. 1. Growth parameters for embryo and placenta at E14.5 and E18.5 from control and superovulated matings. (A) Embryo weight in grams (E14.5: control $n = 17$, superovulated $n = 31$; E18.5: control $n = 10$; superovulated $n = 32$), (B) embryo size (crown rump length) in cm (E14.5: control $n = 17$, superovulated $n = 31$; E18.5: control $n = 10$; superovulated $n = 32$) and (C) placenta weight (*g*) (E14.5: control $n = 16$, superovulated $n = 28$; E18.5: control $n = 10$; superovulated $n = 31$).

2.2. RNA extraction and cDNA synthesis

Whole embryo, placenta or yolk sac was ground to generate a fine powder. Approximately 25–30 mg of tissue was used for RNA extraction using the RNeasy RNA extraction Kit (Qiagen) following the manufacturer's instructions. cDNA synthesis was performed using Superscript II (Invitrogen), random hexamers and 1 μg total RNA. Prior to use for allele-specific expression assays or quantitative RT–PCR, samples were diluted 1/12.5.

2.3. Allele-specific expression assays

Allele-specific expression was carried out on cDNA using a LC Real Time PCR system (Roche Biochemicals). Expression of *H19*, *Snrpn* and *Kcnq1ot1* were assessed as previously described (Fortier et al., 2008). Briefly, specific primers were used to amplify a region of the gene containing a single nucleotide polymorphism between the CD1 and Cast7 strains. Hybridization probes were added to the