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EFFECTS OF PROGESTERONE ON THE EXPRESSION OF GROWTH-RELATED IMPRINTED GENES *Igf2*, *H19* AND *Grb10* IN THE MOUSE PLACENTA

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Summary: Genomic imprinting is an epigenetic process resulting in the monoallelic parent-of-origin-specific expression of a subset of genes in the mammalian genome. Many imprinted genes are expressed during embryo growth and are important for fetal and placental development. Progesterone is a natural steroid hormone involved in the establishment and maintenance of pregnancy. Despite the wide use of progesterone as a protecting agent against miscarriages during human pregnancy, very little is known about its effects on the developing embryo.
In the present work, we investigated the effects of progesterone on the postimplantation embryo development and in particular, the expression of some growth-related imprinted genes in mice. The effect of progesterone was studied after *in vivo* administration (injection of pregnant mice). The expression of the imprinted genes *Igf2*, *H19* and *Grb10* was assessed by quantitative RT-PCR. Special attention was given to the placenta as it is the most likely site where progesterone would exert its effects.

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Key words: Grb10; H19; imprinted genes; mouse placenta; progesterone.

Abbreviations: ART – Assisted reproductive technologies; E – embryo; ICM – inner cell mass; LOS – Large Offspring Syndrome; QPCR – Quantitative RT-PCR.

INTRODUCTION

Genomic imprinting is an epigenetic phenomenon which results in a monoallelic, parental-specific expression of subset of genes in mammals. More than a hundred imprinted genes have been identified in mice and over 50 in humans. Many imprinted genes are crucial for embryo growth and development. After

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birth some are involved in metabolism and some are expressed in the central nervous system, showing major effects on cognition and behavior (Surani et al., 1984; Abramowitz and Bartolomei, 2011). DNA methylation and histone modifications are involved in the establishment of monoallelic expression without altering the genomic sequence. These epigenetic marks are gained in the germline and maintained through mitotic divisions (Reik and Walter, 2001).

Imprinting is believed to evolve as a result of a conflict between maternal and paternal genomes over allocation of resources to the developing embryo. This is in the basis of the kinship theory of genomic imprinting. Indeed, paternal genes tend to stimulate fetal growth, while maternal genes suppress it (More and Haig, 1991). The most likely site of this battle is the placenta as it is the main source of nutrients for the growing embryo. Except the regulation of nutrient allocation, these genes regulate the adaptive response of the placenta to the changing environment (Fowden et al., 2011).

Imprinted genes might be particularly vulnerable to disruption as they are functionally haploid. The genomic imprinting disruptions occurring early in embryonic development can be inherited through many cell cycles into adult tissues and even can cross generations trough germ cells. In recent years an increasing number of studies have demonstrated the ability of different environmental factors to alter imprinted gene expression and a link has been proposed to numerous adult diseases and cancer (Susiarjo M et al., 2013; Young, 2001). Additionally, *in vitro* techniques used in assisted reproductive technologies (ART) might also be associated with deregulation of imprinted genes in mice (Rivera et al., 2007) and can result in abnormally large offspring in ruminants - a condition called Large Offspring Syndrome (LOS). A similar syndrome in humans, Beckwit-Wiedemann syndrome demonstrates alterations in at least two clusters of imprinted genes (Maher and Reik, 2000). Children born from ART have a higher percent of incidence in loss of imprinting syndromes, such as Beckwit-Wiedemann syndrome and Angelman syndrome. ART procedures usually involve ovarian hyperstimulation, culturing and other techniques connected with handling embryos including transplantation, which have been shown to influence imprinted gene expression (Maher et al., 2003; DeBaun et al., 2003).

Additionally, as part of the common ART protocols, progesterone is prescribed at high doses for a few weeks following embryo transfer or even prior to transfer. Progesterone is also frequently prescribed during normal pregnancies as an agent against miscarriages. It is generally considered safe to use during pregnancy, even at high doses and is not believed to affect embryo development. Whether progesterone can alter imprinted gene expression is not clear as very few data are available in literature concerning the possible influence of this steroid hormone. In breast adenocarcinoma *H19* overexpression was significantly correlated with the presence of estrogen and progesterone receptors (Adriaenssens et al., 1998). The same authors showed also that *H19* expression in the mammary gland and the uterus was up-regulated by 17-b-estradiol

and down-regulated by progesterone (Adriaenssens et al., 1999). Progesterone stimulates *Igf2* expression in human osteoblastic cells (Tremollieres et al., 1992). Though considered not to affect embryo development, administration of progesterone in early pregnancy in cattle and sheep was shown to enhance fetal growth (Clemente et al., 2009; Kleemann et al., 1994). In LOS it was noted that transfer of embryos to advanced recipients resulted in significantly longer fetuses then the control group. It is argued that the observed phenomenon is a result of premature increase of progesterone and embryos receive inappropriate developmental signals, which lead to accelerated growth (Young et al., 1998).

The aim of the present study was to elucidate the effects of exogenous progesterone administered *in vivo* on the development of mouse embryos as well as the expression of the growth-related imprinted genes *Igf2*, *H19* and *Grb10* in the placenta.

MATERIALS AND METHODS

Animals

Mice of strain ICR were used in these experiments. Females 10-12 weeks of age were mated to ICR males. The day a vaginal plug was obtained was denoted d0.5. Animals were assigned to two groups – a control group receiving no treatment and a progesterone-treated group. From d4.5 until d8.5 the treated group received daily injections of progesterone 100 µg per mouse (~3.5mg/kg). On d11.5 females were sacrificed by euthanasia (permitted method) and embryos and placentas carefully removed from the uterus. The separated embryos and placentas were weighed and the crown-rump length of embryos and the diameter of placentas were recorded. The number of resorption sites was also recorded. The collected material was frozen until further use.

RNA isolation, reverse transcription and expression quantification

Total RNA was extracted from d11.5 placentas by using GenElute^{tm} Mammalian Total RNA Miniprep Kit (Sigma) according to the manufacturer's instructions. Prior reverse transcription 1µg of isolated RNA was DNaseI (Thermo Scientific) treated. cDNA was prepared by using First Strand Synthesis Kit (Thermo Scientific) and random hexamers. Quantitative RT-PCR was carried out using Maxima SYBR qPCR Master Mix (2x) and PikoReal 96-real time PCR system (Thermo Scientific). QPCR was performed in a 25µl reaction volume. Each reaction contained 12.5µl Maxima SYBR Green Master Mix (2x), 300nM (final concentration) of each primer, PCR grade water and 2.5 µl of cDNA template (125ng). All samples were analyzed in triplicates and no template control was included in each run. PCR cycling program for *H19, Grb10, Igf2 P0, Igf2 P3, ActB* and *Hsp90ab1* genes consisted of 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C. An additional step for final extension was used - 5 min at 72°C. A melting curve analysis was performed to verify the specificity and identity the PCR product. A melting curve program was carried out at 60-95°C with a heating rate of 0.2°C. The investigated transcripts showed high real-time PCR efficiency rates for: *H19* - 1.90; *Grb10*

- 1.92; *Igf2* P0 - 1.94; *Igf2* P3 -1.94; *ActB* – 1.98; and *Hsp90ab1*- 1.99 with good linearity. Primers sequences: *Igf2* P0: forward ATTGACCCAGCCAGC GGATC / reverse - CTGTACTCTAGTC GCTTCGTAG; *Igf2 P3*: forward- CTGGACATTAGCTTCTCCTGTG / reverse- CTGAGGTTGGGTAAG-GAGGC; (Hagègea at al., 2006); *Grb10*: forward- CATGAGATCG T G G T C C A A G T G G / r e v e r s e - ATGCTGCTTTCTTCCAGGTCAGCC *H19*: forward- CCACTACACTACCGCC TAGGA-ATCTGC / reverse-GGTG G G T A C T G G G G C A G C A T T G (Kharroubi at al.,2001); *ActB*: forward – TGGAATCCTGTGGCATCCATGAA / reverse TAAAACGCAG CTCAGTAA CAGTC; *Hsp90ab1*: forward - GCTGGC TGAGGACAAGGAGA / reverse- CGT CGG-TTAGTGGAATCTT CATG (Ficz et al., 2011).

The relative quantification of mRNA levels was determined by normalization against two control genes, *ActB* and *Hsp90ab1*, using the Pfaffl method (Pfaffl, 2001) and the relative expression software tool (REST 2009).

RESULTS

Effects of exogenous progesterone on the expression of the imprinted genes H19, Grb10, Igf2 P0, Igf2 P3 in mouse d11.5 placentas

We analyzed the expression of several growth-related imprinted genes in progesterone-treated d11.5 placentas compared to control placentas. The expression of *H19* was significantly lower (~2 fold) in the treated group compared to control (0.510 relative to the control, $P<0.05$; Fig. 1). The level of *Grb10* expression was also significantly reduced (2-fold) in the treated group (0.496 relative to the control; $P<0.05$; Fig. 1). The levels of expression of *Igf2 P0* placenta specific promoter and the main embryonic *Igf2* promoter *P3* were not significantly different from controls (0.641; $P=0.089$ and 0.509; $P=0.106$ respectively; Fig. 1).

Effect of exogenous progesterone on embryo growth

Our results showed a difference in the average weight of fetuses between the treated and control group. Embryos from

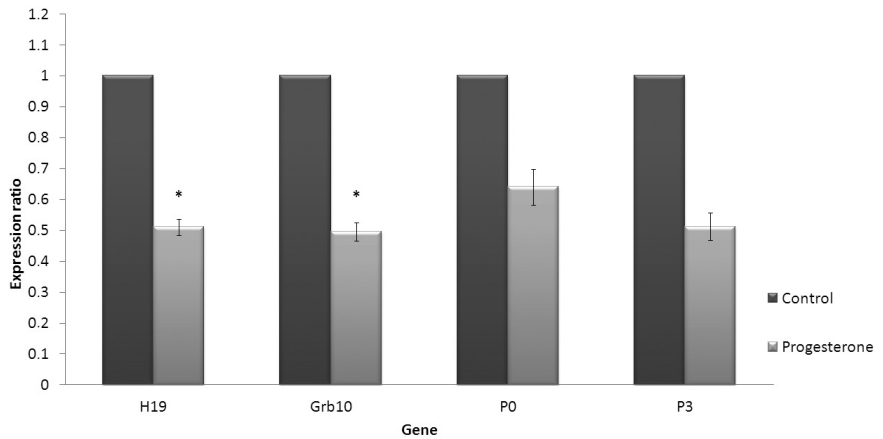

Figure 1. Expression ratios for different genes represented relative to control group mean expressions (adjusted to 1). Dark bars represent control placentas and light bars - treated placentas. Error bars – SEM, *$P<0.05$.

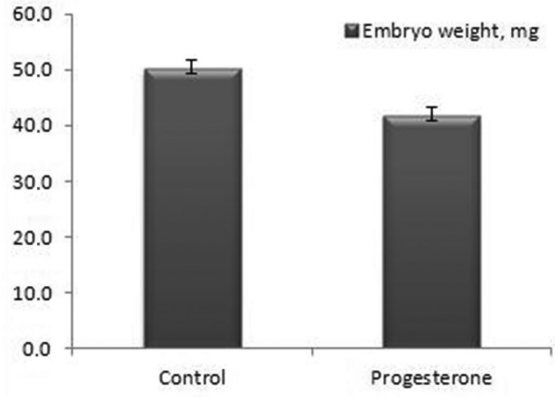

Figure 2. Effect of exogenous progesterone on fetal weight. The mean weights of control group d11.5 embryos and treated group d11.5 embryos were compared (16 embryos per group). Error bars - SEM; $^{**}P<0.001$.

the treated group (Fig. 2) were 16 % lighter then the embryos of the control group (Fig. 2), the difference being statistically significant ($P<0.001$).

DISCUSSION

Our data showed that exogenous progesterone administered to pregnant female mice from d4.5 to d8.5 led to a moderate decrease in the expression of *H19* in the placenta at midgestation (Fig. 1).

H19 is a maternally expressed imprinted gene located in the distal part of chromosome 7 in mice and it codes for untranslated RNA (ncRNA). In *in situ* hybridization experiments *H19* was first detected in extraembryonic tissues as early as 5.5 E, but not in the embryo until day 8.5 E (Poirier et al., 1991). Another research showed that it was expressed from the whole blastocyst, but not from the ICM alone, which again narrows its expression to the trophoectoderm. This localization suggests that *H19* plays an important role in embryo implantation and nutrient transfer across the placenta (Doherty et al., 2000).

In a large number of human cancers *H19* has been shown to be up-regulated. There is a great similarity between tumor and trophoblast invasiveness, but unlike tumor invasion, trophoblast invasion is strictly controlled. Progesterone plays an essential role regulating negatively trophoblast invasion by controlling matrix metalloproteinases and other factors (Halasz and Szekerez-Bartho, 2013). Based on the *H19* localization in the trophoectoderm of the periimplantation embryo and its oncogenic activity, it can be suggested that *H19* is involved in the trophoblast invasion and thus controlled by progesterone. The proposed scenario might account for the observed decrease in the expression of *H19* after administration of exogenous progesterone at perimplantation.

Another imprinted gene that was affected by exogenous progesterone in our study was *Grb10* (Fig. 1). It is located on mouse chromosome 11 and is maternally expressed in the embryo, but paternally expressed in the fetal and adult brain. It encodes a signaling adapter protein that negatively regulates placental and embryonic growth, opposed to *Igf2* that

is a growth enhancer. *Grb10* is shown to regulate growth independently of *Igf2* (Charalambous et al., 2003).

In our study, *Grb10* expression was decreased in the treated group compared to control, while at the same time the treated embryos were smaller, which seemed contradicting with the proposed function of *Grb10*. In fact, the observed decrease was small (~2 fold) and probably didn't affect the weight of the embryos. On the other hand, the placentas didn't show any significant difference in their weights between the two groups (data not shown). The fact that the placentas of the treated group were similar to those of the control group might be a result of *Grb10* being slightly decreased as at the same time *Igf2* expression of P3 promoter (though not statistically significant) as well P2 (unpublished results) were also decreased. It can be proposed that the effects of the genes were additive.

The modest retardation of the treated embryos (Fig. 2) compared to control could serve as a verification of the obtained results, and demonstrate that the placental function was altered as a result of progesterone treatment. A similar inhibitory effect on fetal growth has been observed in transgenic mouse models overexpressing *hIGFBP1*. IGF (insulin growth factor) binding protein-1 modulates the actions of IGF2 on the trophoblast cells and thus it is important for the regulation of placental development and fetal growth. The expression of *Igfbp-1* is largely regulated by progesterone (Crossey et al., 2002). Whether this is the case with our treated group embryos and what is the expression status of the imprinted genes reported herein remains to be elucidated.

In conclusion, exogenous progesterone administered *in vivo* to pregnant female mice at periimplantation showed a moderate effect on the expression of some imprinted genes in the mouse placenta. Exogenous progesterone had also an effect on the embryo as embryos were significantly smaller then the control ones. Probably the observed result was not a direct effect of progesterone on the developing embryo, but rather indirect as a consequence of altered intrauterine environment.

Further studies are needed to elucidate in detail the effects and mechanisms of action of this steroid hormone on the growth and development of the embryo.

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REFERENCES

Abramowitz Lara K, Bartolomei Marisa S, 2012. Genomic imprinting: recognition and marking of imprinted loci. *Current Opinion in Genetics and Development*, 22(2): 72–8.

Adriaenssens E, Dumont L, Lottin S, Bolle D, Leprêtre A, Delobelle A, Bouali F, Dugimont T, Coll J & Curgy JJ, 1998. H19 overexpression in breast adenocarcinoma stromal cells is associated with tumor values and steroid receptor status but independent of p53 and Ki-67 expression. *Am J Pathol*, 153: 1597–1607.

Adriaenssens E, Lottin S, Dugimont T, Fauquette W, Coll J, Boilly B, Curgy JJ, 1999. Steroid hormones modulate H19 gene expression in both mammary gland and uterus. *Oncogene*, 18: 4460–4473.

Charalambous, M., Smith, F. M., Bennett, W. R., Crew, T. E., Mackenzie, F. and Ward, A, 2003. Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism. *Proc Natl Acad Sci USA*, 100: 8292–8297.

Clemente, M., de La Fuente, J., Fair, T., Al Naib, A., Gutierrez-Adan, A., Roche, J. F., Rizos, D., and Lonergan, P, 2009. Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? *Reproduction*, 138: 507–517.

Crossey PA, Pillai CC, Miell JP, 2002. Altered placental development and intrauterine growth restriction in IGF binding protein-1 transgenic mice. *J Clin Invest*, 110: 411–418.

DeBaun MR, Niemitz EL, Feinberg AP, 2003. Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am J Hum Genet*, 72: 156-160.

Doherty A.S., M.R. Mann, K.D. Tremblay, M.S. Bartolomei, R.M. Schultz, 2000. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol Reprod*, 62: 1526–1535.

Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W, 2011. Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature*, 473(7347): 398–402.

Fowden, A.L., Coan, P.M., Angiolini, E., Burton, G.J., Constancia, M, 2011. Imprinted genes and the epigenetic regulation of placental phenotype. *Prog Biophy Mol Biol*, 106: 281–288.

Hagègea R, Nassera M, Webera L, Milligana N, Aptela C, Jacqueta RA, Drewellb L, Dandoloc MA, Suranid G, Cathalaa T, Forné, 2006. The 3' portion of the mouse *H19* Imprinting-Control Region is required for proper tissue specific expression of the *Igf2* gene. *Cytogenet Genome Res*, 113: 230–237.

Halasz M, J Szekeres-Bartho, 2013. The role of progesterone in implantation and trophoblast invasion. *J Reprod Immunol*, 97 (1): 43–50.

Kharroubi El, Piras G, Stewart CL, 2001. DNA Demethylation reactivates a subset of imprinted genes in uniparental mouse embryonic fibroblasts. *J Biological Chemistry*, 276(12): 8674–8680.

Kleemann DO, Walker SK, Seamark RF, 1994. Enhanced fetal growth in sheep administered progesterone during the first three days of pregnancy. *J Reproduction and Fertility*, 102: 411–417.

Maher ER, Reik W, 2000. Beckwith-Wiedemann syndrome: imprinting in clusters revisited. *J Clin Invest*, 105: 247–252.

Maher ER, Brueton LA, Bowdin SC, Luharia A, Cooper W, Cole TR, Macdonald F, Sampson JR, Barratt CL, Reik W, Hawkins MM, 2003. Beckwith-Wiedemann syndrome and assisted reproduction technology (ART). *J Med Genet*, 40: 62–64.

Moore T., D. Haig, 1991. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet*, 7: 45–49.

Pfaffl MW, 2001. A new mathematical model for relative quantification in real-time RT PCR. *Nucleic Acids Res.*, 29, 9:e45.

Poirier F, Chan CT, Timmons PM, Robertson EJ, Evans MJ, Rigby PW, 1991. The murine H19 gene is activated during embryonic stem cell differentiation in vitro and at the time of implantation in the developing embryo. *Development*, 113: 1105–1114.

Reik W, Walter J, 2001. Genomic imprinting: parental influence on the genome. *Nat Rev Genet*, 2: 21–32.

Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM, Bartolomei MS, 2008. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet*, 17: 1–14.

Surani MA, Barton SC, Norris ML, 1984. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature*, 308: 548–550.

Susiarjo M, Sasson I, Mesaros C, Bartolomei MS, 2013. Bisphenol A Exposure Disrupts Genomic Imprinting in the Mouse. *PLoS Genet* 9(4): e1003401.

Tremollieres, F.A., Strong, D.D., Baylink, D.J., and Mohan, S, 1992. Progesterone and promegestone stimulate human bone cell proliferation and insulin-like growth factor-2 production. *Acta Endocrinol*, 126: 329–337.

Young LE, Sinclair KD, Wilmut I, 1998. Large offspring syndrome in cattle and sheep. *Rev Reprod*, 3: 155–163.

Young LE., 2001. Imprinting of genes and the Barker hypothesis. *Twin Res*, 4: 307–317.