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Upregulation of *Ugt1a* genes in placentas and fetal livers in a murine model of assisted reproduction

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Abstract

Genes from *Ugt1a* family in placenta and fetal liver are responsible for hormone, nutrient and chemical balance during pregnancy. Assisted reproduction technologies (ART) *i.e.* intracytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF) alter steroid homeostasis in pregnancy through increased glucuronidation. Here we show that ART (particularly ICSI) upregulates *Ugt1a1*, *1a2*, *1a6* and *1a9* expression in murine placentas and fetal livers with higher mRNA related to lower progesterone (*1a1*) and cholesterol (*1a2*, *1a6*) in placentas. Greater steroid clearance in ART through transcriptional upregulation of *Ugt1a* in the placental-fetal unit may decrease the availability of essential molecules, mediating negative reproductive outcomes.

Keywords

in vitro fertilization; intracytoplasmic sperm injection; placenta; fetal liver; UGT; assisted reproduction; detoxification; endocrinology; pregnancy

Introduction*

During pregnancy, endocrine status is balanced by the collective actions of maternal and fetal organs and the placenta. Therefore, enzymes that have combined pharmacologic,

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Authorship Contributions:

Research design: Collier, Ward

Conducted experiments: Collier (ELISA, western analysis), Milam (q-RT-PCR), Rougée (Western blotting), Sugawara (XY genotyping), Yamauchi (ART, animal husbandry, tissue collection), Ward (animal husbandry, tissue collection)

Performed data analysis: Collier, Milam

Wrote or contributed to writing/editing of the manuscript: Collier, Milam, Rougée, Ward

Other: Collier and Ward acquired funding for this research.

*Abbreviations: assisted reproduction technologies: ART, intracytoplasmic sperm injection: ICSI, *in vitro* fertilization: IVF, UGT/*Ugt*: UDP-glucuronosyl transferase

toxicologic, nutritive and endocrine roles may be critical in regulating fetal development [1]. The UDP-glucuronosyltransferases (UGT/*Ugt* in humans and mice, respectively) are an example of such enzymes.

The UGTs are a superfamily divided into 1A, 2A, 2B and 3A sub-families. In mice, *Ugt1a*, *2a*, *2b* and *3a* are expressed in placenta [2]. Although murine fetal livers show extensive glucuronidation activity [3], hepatic expression of specific *Ugt* isoforms has not yet been described. In humans, multiple active UGT1A and 2B isoforms are expressed in placenta [4-6], and while most isoforms are expressed in fetal liver, activities are low or absent [7-10]. Based on these characteristics, glucuronidation in the placenta is likely critical for chemical, hormone and nutrient disposition during pregnancy in both mice and humans and in this specific study we have focused on the *Ugt1a* subfamily.

Recently, we have demonstrated that steroid transit to the fetus is dysregulated in murine pregnancies conceived with assisted reproduction technologies (ART), with no changes in steroidogenesis, but greater glucuronidation in the placental and fetal units [11, 12]. Increased glucuronidation is also associated with removal of progesterone, other hormones and oxidative stress in the placental-fetal unit [13]. Here we demonstrate that transcriptional upregulation of *Ugt1a* isoforms is, in part, responsible for this phenomenon. Because hormones maintain pregnancy, the consequences of inappropriate steroid elimination by *Ugt* may be dire.

Materials and Methods

The methods for generating mouse pregnancies (ART and normal mating), pregnancy outcomes, and tissue processing have been previously described [12]. Briefly, B6D2F1 mice (C57BL/6 × DBA/2) and CD-1 strains were used for mating and as sperm and oocytes donors for IVF and ICSI, and CD-1 mice were used as surrogate mothers and vasectomized males for embryo transfer. Three dams were used for each of normal, IVF and ICSI producing 26, 37 and 25 detuses respectively. Tissues were collected at gestational day 18.5 by caesarian section, placed on ice for not more than 30 minutes then snap frozen and stored at -80 °C until use. A subset of samples were used, with n = 9 for mating, IVF and ICSI placentas (3 from each litter, at least 1 female from each) and n = 6 for fetal livers (2 from each litter: 1 male, 1 female). These studies were approved by the Institutional Animal Care and Use Committee at the University of Hawaii.

Fetal and placental sex determination

Tissues were sexed by genotyping for X chromosome gene *Smcx* and Y chromosome gene *Smcy* using one primer pair (F: TGAAGCTTTTGCTTTGAG; R: CCGCTGCCAAATTCCTTGG) amplifying both genes and yielding different size products (*Smcx*: 330 bp and *Smcy*: 300 bp). DNA extraction, PCR and electrophoresis were performed as previously described [14]

Quantitative RT-PCR for *Ugt1a* expression

Specific *Ugt1a* primers were synthesized by Idaho Technology Inc. (Salt Lake City, UT) from previously published sequences [15-18]. RNA was extracted using Qiagen RNeasy kits (Valencia CA) and assessed for purity with Nanodrop 2000 (Wilmington, DE). Reverse transcription was performed according to Promega's instructions (Madison, WI). Quantitative RT-PCR was performed using 1 ng cDNA, 500 nM of each *Ugt1a* or 18S rRNA (control) forward and reverse primers, nuclease-free water and SYBR Green PCR mastermix on an ABI7900HT. Quantification was performed relative to 18S using the method of Pfaffl [19]. Positive control for all isoforms was female mouse liver except

Ugt1a2 (female mouse kidney). Fold changes in mRNA were generated by comparing relative transcript levels from each ART placenta or fetal liver, to average transcript levels in naturally conceived tissues. For comparison of *Ugt* mRNA to sex steroids, transcripts in each sample relative to positive control tissues were plotted against steroid levels in the same tissue.

Western Blot

Proteins from fetal livers/placentas from each dam were pooled, producing three pools of liver and three pools of placenta for each of normal mating, ICSI and IVF. Protein lysates (15 µg placenta, 10 µg fetal liver, and 10 µg positive control (pooled human liver, (Xentotech, KS) and recombinant human UT1A1 (Invitrogen, CA)) were electrophoresed on denaturing 10% acrylamide gels with semi-dry transfer to PVDF. Total *Ugt1a* detection occurred with primary antibody (1:100 polyclonal UGT1A, SCBT, CA), donkey-anti-rabbit biotin (1:10,000, 2 hr, Jackson, PA) and streptavidin-HRP (1:3000, 1 hr, GE Healthcare, NJ), before chemiluminescent detection (5 min fetal liver, 1 hr placenta). Sizing was in comparison to recombinant protein and Rainbow Marker™. Ratiometric area:density analyses were performed with Image J (<http://rsbweb.nih.gov/ij/>), using pooled human liver as the reference.

Quantification of steroids

Cholesterol, estrone, estradiol and progesterone levels were quantified in tissue lysates using commercial ELISA (Alpco Diagnostics, Salem, NH) previously validated by us [12].

Statistical Analyses

Non parametric t-tests and Spearman's correlations were used since data was not normally distributed according to D'Agostino and Pearson's test (GraphPad Prism, San Diego CA).

Results and Discussion

Mouse placentas from pregnancies conceived by mating and ART constitutively expressed *Ugt1a1*, *1a2* and *1a6* in all tissues tested (9/9) but not *1a7*, *1a8*, *1a9* or *1a10* which could not be amplified in less than 32 cycles, despite successful amplification of these latter isoforms from mouse liver and kidney. Expression was deemed "absent" if cycles for amplification were greater than 32. The *Ugt1a5* isoform was not tested. Results differ somewhat from the findings of Buckley and Klaassen (2007) who showed constitutive placental expression of *Ugt1a5*, *1a6*, *1a8*, *1a9* and *1a10*, in C57BL/6 mice, although the authors expressed reservations regarding very low expression of latter three isoforms. Contrasting results are likely due to the different mouse strain used and/or differences in method sensitivity.

Placentas from pregnancies achieved by IVF and ICSI showed increase in expression of *Ugt1a1* (61-fold for IVF and 106-fold for ICSI, $P < 0.001$) as compared to placentas from normally mated mice, (Figure 1A). Expression of *Ugt1a2* was increased 14-fold ($P < 0.01$) and 22-fold ($P < 0.001$) in IVF and ICSI, respectively, while *Ugt1a6* increased 8-fold, both $P < 0.05$ (Figure 1A). In contrast, for protein, while a trend of increasing placental *Ugt1a* protein was observed, only ICSI has significantly higher protein than normal placentas ($P < 0.05$, Figure 1B, C).

Fetal liver from normally conceived pregnancies expressed *Ugt1a1*, *1a2* and *1a6*, in all tissues tested (6/6 samples). Evidence for constitutive expression of *Ugt1a9* was observed in 2/6 fetal livers. While ART did not significantly alter fetal liver *Ugt1a* expression compared to normally conceived fetal livers, consistent 2-4 fold induction of *Ugt1a* expression was

observed (Figure 1D). Moreover, *Ugt1a1* expression was significantly higher in IVF compared to ICSI while *Ugt1a6* it was greater in ICSI than IVF (Figure 1D). This was echoed by significant increases in *Ugt1a* protein in IVF ($P < 0.01$) and ICSI ($P < 0.001$) compared to normal mating as well as ICSI vs. IVF ($P < 0.05$) measured by area-density analysis (Figures 1E, F)

Sexual dimorphism and hormone-dependent expression of *Ugt* genes has been documented in adult mouse tissues and in humans [20]. Here, no relationship between expression of *Ugt* isoforms in murine fetal livers and placentas and the genetic sex of the fetus was observed. However, in placenta *Ugt1a1* mRNA expression was negatively correlated with progesterone levels ($P = 0.007$, Figure 2A) while *Ugt1a2* and *Ugt1a6* were negatively correlated with cholesterol ($P = 0.018$, Figure 2B and $P = 0.021$ Figure 2C), suggesting that levels of these molecules are associated with *Ugt* expression. Negative regulation of *Ugt1a1* by progesterone in the placenta is somewhat supported by the findings of Buckley and Klaassen [20] who demonstrated that *Ugt1a2* in mouse kidney is negatively regulated by testosterone. These authors (and others) have also demonstrated that *Ugt* expression in the mouse is highly tissue- and isoform-dependent, with androgens (testosterone) regulating *Ugt1a2* expression [20].

The maternal environment of the pregnancies (BD62F1 mothers for normally mated vs. CD1 mothers for IVF and ICSI) cannot be excluded as responsible for differences observed. However, this unlikely to be a major factor in our results, because differences between IVF and ICSI (same maternal environment) were also observed. Moreover, with the exception of the decidua; the placenta is an organ of fetal origin and all of the fetuses were the same genetic background. In summary, *Ugt1a* expression in murine placentas and fetal livers is upregulated in ART, providing at least part of the mechanism for our previously reported observations of decreased levels of steroids and increased steroid glucuronides in the placental-fetal unit when ART is used [11, 12]. It is also possible that other *Ugt* subfamilies, namely *Ugt2a*, *Ugt2b* or *Ugt3a* could likewise be upregulated. Moreover, as the amount of cholesterol and progesterone in placentas increased, transcripts levels of *Ugt1a1*, *1a2* and *1a6* decreased. If the same mechanism occurs in humans, it may explain higher rates of pre-term birth and small-for-gestational age babies in ART due to excessive removal of steroids and nutrients, providing a target to improve of birth outcomes.

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Figure 1. Increases in *Ugt1a* mRNA and protein expression in mouse placentas and fetal livers caused by ART

1A: Expression and fold increases in *Ugt1a* mRNA expression in placentas from ART pregnancies compared to placentas from pregnancies conceived by mating. 1B: Increasing expression of UGT1A protein in placentas shown as area density analysis of western blots (1C). 1D: Expression and fold increases in *Ugt1a* mRNA expression in fetal livers from ART pregnancies compared to fetal livers from pregnancies conceived by mating. 1E: Increasing expression of UGT1A protein in fetal livers shown as area density analysis of western blots (1F). Bars are means $\pm$ SEM of $n = 9$ (placenta) or $n = 6$ (fetal liver). * = $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. tissues from pregnancies conceived by mating. (—) designates significance between IVF and ICSI. One representative blot from $n = 3$ each is presented.

M=mating, V-IVF, C=ICSI.

Figure 2. Expression of *Ugt1a* mRNA in placentas is negatively correlated with endogenous hormones

2A: *Ugt1a1* is negatively correlated with progesterone, ($r = 0.52$, $P = 0.007$). 2B: *Ugt1a2* is negatively correlated with cholesterol ($r = 0.45$, $P = 0.018$). 2C: *Ugt1a6* is negatively correlated with cholesterol, ($r = 0.044$, $P = 0.02$). Solid lines (-) show the linear regression, dashed lines (---) represent the 95 % confidence interval of the regression. Spearman's correlations were performed for 3 isoforms *Ugt1a1*, *1a2* and *1a6* against four hormones; cholesterol, progesterone, estradiol and estrone, with results presented only for significant findings.