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Igf2 deletion alters mouse placenta endocrine capacity in a sexually dimorphic manner

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Abstract

The placenta regulates materno-fetal nutrient transfer and secretes hormones that enable maternal physiological support of the pregnancy. In mice, these functions are performed by the labyrinth (Lz) and junctional (Jz) zones, respectively. Insulin-like growth factor 2 (*Igf2*) is an imprinted gene expressed by the conceptus that is important for promoting fetal growth and placenta formation. However, the specific role of *Igf2* in the Jz in regulating placental endocrine function and fetal development is unknown. This study used a novel model to investigate the effect of conditional loss of *Igf2* in the Jz (*Jz-Igf2*UE) on placental endocrine cell formation and the expression of hormones and IGF signaling components in placentas from female and male fetuses. *Jz-Igf2*UE altered gross placental structure and expression of key endocrine and signaling genes in a sexually dimorphic manner. The volumes of spongiotrophoblast and glycogen trophoblast in the Jz were decreased in placentas from female but not male fetuses. Expression of insulin receptor was increased and expression of the MAPK pathway genes (*Mek1*, *P38α*) decreased in the placental Jz of female but not male fetuses. In contrast, expression of the type-1 and -2 IGF receptors and the MAPK pathway genes (*H-ras*, *N-ras*, *K-ras*) was decreased in the placental Jz from male but not female fetuses. Expression of the steroidogenic gene, *Cyp17a1*, was increased and placental lactogen-2 was decreased in the placenta of both sexes. In summary, we report that *Jz-Igf2*UE alters the cellular composition, IGF signaling components and hormone expression of the placental Jz in a manner largely dependent on fetal sex.

Key Words

- ▶ insulin-like growth factor
- ▶ placenta
- ▶ hormones
- ▶ endocrine cells
- ▶ trophoblast

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Introduction

Pregnancy success depends upon the appropriate allocation of nutrients between the fetus for growth and development and the mother to sustain the pregnancy and subsequent lactation. Failure to achieve this materno-fetal nutrient balance can lead to pregnancy complications including abnormal birth weight and gestational diabetes (Sferruzzi-Perri *et al.* 2013a, 2020, Plows *et al.* 2018). Inappropriate nutrient allocation during gestation can also program offspring for life-long health complications including metabolic and cardiovascular dysfunction

(Fowden *et al.* 2006, Gluckman *et al.* 2008, Camm *et al.* 2018). The placenta is a main determinant of materno-fetal nutrient allocation. It controls nutrient transport between the mother and fetus and secretes hormones which adapt maternal physiology to support fetal nutrient supply and growth (Sferruzzi-Perri & Camm 2016, Napso *et al.* 2018). Previous work has largely focused on understanding the importance of placental transport function in the context of pregnancy outcome (Fowden *et al.* 2006, Desforges & Sibley 2009, Dimasuay *et al.* 2016, Gaccioli & Lager 2016).

However, much less is known about the role and regulation of placental endocrine function in pregnancy.

Information on the role of placental hormones has principally been derived from association studies between maternal serum hormone levels or placental hormone gene expression and fetal growth in humans (Mirlesse *et al.* 1993, McIntyre *et al.* 2000, Chellakooty *et al.* 2004, Koutsaki *et al.* 2011), as well as studies administering or genetically manipulating the expression of placental hormones and their receptors in experimental animals or cultured cells (Nogues *et al.* 1997, Carter 2012, Burton *et al.* 2016, Napso *et al.* 2018, Sferruzzi-Perri *et al.* 2020). Placental hormones, such as growth hormone variant and members of the placental lactogen/prolactin gene family reduce whole body insulin sensitivity of the mother by altering the expression of insulin signaling components in the skeletal muscle and white adipose tissue (Jarrett *et al.* 1984, Barbour *et al.* 2002, 2004). Similarly, progesterone induces insulin resistance in the liver, skeletal muscle and adipose tissue by inhibiting the phosphoinositide 3-kinase (PI3K) signaling pathway (Sutter-Dub & Dazey 1979, Leturque *et al.* 1984, Wada *et al.* 2010). In contrast, estrogen increases maternal insulin sensitivity as loss of aromatase (*Cyp19*), a key rate-limiting gene for estrogen production, leads to glucose intolerance and decreased insulin sensitivity (Takeda *et al.* 2003). Prolactins and placental lactogens also increase the number, size and insulin secretion of beta cells in the pancreas to balance the level of insulin resistance in the mother during pregnancy (Vasavada *et al.* 2000, Brelje *et al.* 2004, Weinhaus *et al.* 2007).

In addition to mediating systemic physiological adaptations in the mother during pregnancy, placental hormones act locally to promote angiogenesis and immunomodulation that are important for fetal development. Pregnancy-specific glycoproteins (PSGs) are synthesised by the placenta and induce the secretion of anti-inflammatory cytokines by maternal leukocytes to promote a tolerogenic decidual environment (Snyder *et al.* 2001). They also promote endothelial tube formation and the expression of angiogenic growth factors (Blois *et al.* 2012).

The mouse is a useful species for studying placental endocrine function. In mice, the endocrine and transport functions of the placenta are carried out in the structurally discrete junctional zone (Jz) and labyrinth zone (Lz), respectively (Coan *et al.* 2006). This differs to humans, where both functions are performed by the placental syncytiotrophoblast (Dearden & Ockleford 1983),

preventing evaluation of their importance in isolation. In mice, the placental Jz forms under genetic instruction distinct from the Lz (Simmons & Cross 2005) and the two zones can be physically separated (Sferruzzi-Perri *et al.* 2009). The Jz is composed of three major endocrine cell types: spongiotrophoblasts (SpT), glycogen cells (GC) and trophoblast giant cells (TGC). Murine GC are considered to be analogous to the human placental extravillous cytotrophoblast, which also accumulate glycogen (Wislocki & Bennett 1943, Georgiades *et al.* 2002). SpT, GC and TGC vary in the repertoire of hormones they express (McLellan *et al.* 2005, Simmons *et al.* 2008, LaVoie & King 2009) and within the mouse placenta, they are the only cells that express the gene; trophoblast-specific protein alpha (*Tpbpa*) (Lescisin *et al.* 1988). While there are some differences in the hormones expressed in mouse and human placenta, many mouse-specific hormone genes are structurally similar to those in human and perform similar functions (Soares 2004, Carter 2012). Moreover, many genes, proteins and cellular pathways governing placental formation and function overlap in mice and humans (Cox *et al.* 2009, 2011).

Insulin-like growth factor 2 (*Igf2*) is a paternally expressed imprinted gene that is highly expressed in the fetus and placenta of mice and humans (DeChiara *et al.* 1991, Sferruzzi-Perri *et al.* 2011a). In the human placenta, *IGF2* is highly expressed by the villous and extravillous cytotrophoblasts during the first trimester and the extravillous cytotrophoblasts during the third trimester (Brice *et al.* 1989, Han *et al.* 1996, Thomsen *et al.* 1997). In mice, *Igf2* is expressed by the placental trophoblast and fetal vasculature from D9.5 (Redline *et al.* 1993). Within the Jz, *Igf2* expression is initially strongest in the SpT but from ~D12.5, *Igf2* is restricted to the GC (Redline *et al.* 1993). *IGF2* is a small 7.5 kDa protein that promotes mitogenesis, metabolism, differentiation and survival of multiple cell types (Jones & Clemmons 1995, Curchoe *et al.* 2005, Guvakova 2007). Its anabolic actions are mostly mediated via binding to the type-1 IGF receptor (IGF1R) or insulin receptor (INSR), which typically leads to activation of the PI3K-AKT or RAS-MAPK-ERK pathways (McClain 1991, Forbes *et al.* 2008, Kent *et al.* 2012, Sferruzzi-Perri *et al.* 2017, Sferruzzi-Perri 2018). However, *IGF2* can also bind to the type-2 IGF receptor (IGF2R), which can result in its degradation (Oka *et al.* 1985, McClain 1991, Sferruzzi-Perri *et al.* 2017, Sferruzzi-Perri 2018).

IGF2 is an important growth regulator that has been implicated in the development of human fetal growth disorders, namely Silver-Russell syndrome (SRS)

and Beckwith–Wiedemann syndrome (BWS), which are characterised by fetal growth restriction and fetal overgrowth, respectively. Aberrant methylation of the *IGF2* imprinting control region (ICR1) has been implicated in both conditions, with hypomethylation of ICR1 reported in 37–63% of SRS cases and hypermethylation of ICR1 reported in up to 10% of BWS cases (reviewed in Öunap 2016). Moreover, expression of *IGF2* in human chorionic villus samples at week 11–13 of gestation has been positively correlated with birth weight (Demetriou *et al.* 2014) and exogenous *IGF2* induced proliferation of primary human trophoblast *in vitro* (Chen *et al.* 2016). In mice, constitutive deletion of *Igf2* results in fetoplacental growth restriction (DeChiara *et al.* 1991) in association with reduced Jz formation, GC abundance and glycogen content (Lopez *et al.* 1996). In addition, wild-type mouse dams carrying litters that lack the placental Lz-specific *Igf2* isoform (*Igf2P0*) have altered body weight and increased circulating insulin, corticosterone and glucose concentrations when fed *ad libitum* and/or undernourished during pregnancy (Sferruzzi-Perri *et al.* 2011b). Finally, *Igf2* and PI3K are correlated with the expression of placental hormone genes in the mouse placenta (Ishida *et al.* 2007, Sferruzzi-Perri *et al.* 2016). Together, these findings suggest that *Igf2* may play a role in regulating placental endocrine function and consequently maternal metabolic state, but the mechanism through which this is achieved is unclear. However, to date our understanding has been limited to global or Lz specific manipulations of *Igf2* in mice.

In this study, we generated a novel mouse model in which *Igf2* is specifically knocked-out in the placental Jz (Jz-*Igf2*UE) using the *Tpbpa*Cre line, which is exclusively expressed in Jz cells (Simmons *et al.* 2007). This enabled us to explore the role of Jz-derived *Igf2* on the endocrine phenotype of the mouse placenta, including cellular composition of the Jz and hormone expression. As the placenta has been shown to exhibit sexually dimorphic responses to environmental and genetic manipulations (Rosenfeld 2015, Kalisch-Smith *et al.* 2017, Barke *et al.* 2019, Napso *et al.* 2019), analyses were conducted separately for males and females fetuses.

Materials and methods

Animal housing and time mating

The University of Cambridge Animal Welfare and Ethical Review Body approved all experiments carried out under the UK Home Office Animals (Scientific Procedures) Act

1986. Homozygous *Tpbpa*Cre females (Simmons *et al.* 2007) (8–10 weeks) were time-mated with heterozygous *Igf2*Flox males (Haley *et al.* 2012) to generate litters with control and Jz-*Igf2*UE fetuses. Both strains were on a C57BL/6NCrl (Charles River) background for >10 generations. Mice were housed under standard 12 h light:12 h darkness photocycle with 21% oxygen at 22°C and *ad libitum* access to water and standard chow diet (Rat and Mouse No.3; Special Dietary Services, Witham, UK).

Tissue collection

Copulatory plug detection was defined as pregnancy day (D)1. On D16 of gestation, dams were killed by cervical dislocation. D16 was selected for analysis as the placental Jz is at its maximal size (Coan *et al.* 2006) and mouse dams are most insulin resistant and glucose intolerant compared to non-pregnant state (Musial *et al.* 2016). Fetuses and placentas were weighed. Fetal tails were collected to determine *Igf2*Flox genotype (FPrimer 5'-TTACAGTTCAAAGCCACCACG-3', RPrimer: 5'-GCCAAAGAGATGAGAAGCACC-3') and sex by *Sry* genotyping (FPrimer: 5'-GTGGGTTCTGTCCCACTGC-3', RPrimer: 5'-GGCCATGTCAAGCGCCCCAT-3' using FPrimer: 5'-TGGTTGGCATTATCCCTAGAAC-3', RPrimer: 5'-GCAACATGGCAACTGGAAACA-3' as a PCR autosomal gene control) using PCR and agarose gel electrophoresis. Placentas were bisected and one half was separated into Jz and Lz and snap frozen for gene expression analysis. The other half was either fixed in 4% paraformaldehyde for histological assessment or frozen for glycogen concentration analysis. The placentas chosen for analysis from each group were selected on the basis of being the closest to the mean placental weight of each litter.

Histological analysis

Fixed placentas were paraffin embedded, sectioned exhaustively at 8 µm with the first 20 midline placental sections kept for analysis and then every 20th section stained with hematoxylin and eosin ($n=8$ /sex/genotype, from 8 litters). The placental mid-line was determined by the presence of all three placental layers (decidua basalis (Db), Jz and Lz) and a complete chorion in the section. Images were captured using a NanoZoomer 2.0-RS (Hamamatsu, Hamamatsu City, Japan) and gross placental structure analysed using the newCAST System (Visiopharm, Hørsholm, Denmark). The proportion

of Db, Jz and Lz in each section was determined using point counting and values were multiplied by placental weight to provide an estimated volume of each region (Coan *et al.* 2004). The first complete mid-line placental section was used to quantify the proportion of different Jz and Db cells. These comprised SpT, GC, TGC, decidual stromal cells (Db_S), decidual glycogen cells (Db_GC) and decidual vessels (Db_V). When multiplied by the corresponding Jz or Db volume, this provided estimated volumes of each cell type within the Jz and Db. The size of 100 individual Jz glycogen cells was measured in a mid-line placental section, using NDP.view2 viewing software (Hamamatsu).

Periodic acid-Schiff (PAS) stain

Sections ($n=2$ /sex/genotype, across 8 litters) were stained with PAS to visualise placental glycogen accumulation. Sections were dewaxed, rehydrated and incubated in 0.5% periodic acid (Thermo Fisher Scientific) for 5 min, rinsed with distilled water, stained with Schiff's reagent (Sigma-Aldrich) for 15 min and rinsed in distilled water. Slides were counterstained with hematoxylin, dehydrated and mounted.

In situ hybridisation

In situ hybridisation was used to assess the spatial expression of *Igf2*, *Prl8a8* and *Tpbpa* genes. *Tpbpa* probe generation has been previously described (Lescisin *et al.* 1988). Novel probes for *Prl8a8* and *Igf2* were generated by PCR amplification of a 512bp region of *Prl8a8* (primer sequences: 5'-CGGCGGGATGAGATTGTTT-3', 5'-GCAATGAACTTTGTGGAGCG-3') and a 507bp region of *Igf2* (primer sequences: 5'-CTCATCTCTTTGGCC TTCGC-3', 5'-TCACTGATGGTTGCTGGACA-3') from placental cDNA and cloning into the pDrive vector system (Qiagen). Digoxigenin labeled riboprobes were generated from linearised plasmid template using the DIG RNA Labelling mix (Roche).

In situ hybridization was performed with probe hybridisation at 60°C overnight, as previously described (Rakoczy *et al.* 2017). BM-Purple alkaline phosphatase substrate aided staining development and Nuclear Fast Red (Sigma-Aldrich) was used to counterstain sections before mounting. Images for sense probes of *Igf2* and *Tpbpa* are shown in Supplementary Fig. 1A and B (see section on [supplementary materials](#) given at the end of

this article). The integrity of the Jz boundaries to the Db and Lz (Db/Jz and Jz/Lz boundaries) was qualitatively assessed using sections stained for *Tpbpa* and *Prl8a8* by *in situ* hybridisation ($n=4$ per sex/genotype). The amount of Jz interdigitation into the Lz was also qualitatively assessed by ranking samples from least to most interdigitated.

Immunohistochemistry

Placenta sections were dewaxed and rehydrated. Antigen retrieval with sodium citrate buffer (0.01 M sodium citrate, 0.05% Tween 20, pH 6) was performed at 80°C for 1 h. Sections were blocked (10% goat serum, 1% BSA in PBS) for 1 h at room temperature before incubation with anti-caspase 3 (Cell Signalling, Cleaved Caspase-3 (Asp175) 9661; 1:200 dilution in 1% BSA/PBS) overnight at 4°C. Sections were incubated with goat-anti-rabbit secondary antibody (Abcam, ab6720; 1:1000 dilution in 1% BSA/PBS) for 1 h at room temperature and streptavidin-horseradish peroxidase (Rockland, Limerick, PA, USA, S000-03, 1:250 dilution in PBS) for 1 h at room temperature. Sections were stained with 3,3'-Diaminobenzidine (Abcam) for 5 min and counterstained with hematoxylin before dehydrating and mounting. The number of caspase positive cells within the Db and Jz of scanned slides was counted in each section.

Placental gene expression

RNA was extracted from separated placental zones ($n \geq 8$ /sex/genotype, from 8 litters) using the RNeasy Plus Mini Kit (Qiagen) and reverse transcribed using the High Capacity cDNA RT Kit minus RT inhibitor (Applied Biosystems) following manufacturer's instructions. Samples were analysed in duplicate using MESA Blue SYBR (Eurogentec, Seraing, Belgium) and primers synthesised by Sigma-Aldrich on a 7500 fast real-time PCR machine (Applied Biosystems). Cycling conditions were as follows: 3 min at 95°C then 40 cycles of: 30 s at 95°C, 30 s at 57°C and 90 s at 72°C. Primer sequences were designed or sourced from published papers (Supplementary Table 1). Primers were verified to have an efficiency >80% and yield a PCR product of the correct size and sequence. The expression of each gene of interest was normalized to the geometric mean expression of the reference genes *Hprt* and *Ywhaz*, which were unaffected by sex or genotype.

Glycogen assay

Placental glycogen content was indirectly measured ($n \geq 7$ /sex/genotype, across $n \geq 7$ litters), using amyloglucosidase as described (Sferruzzi-Perri *et al.* 2013b).

Statistics

As part of our initial quality control check, a Grubb's test was performed as standard on all datasets to identify any outliers (using the Prism Grubbs' test, GraphPad Software Inc.), which were removed prior to statistical analysis. For the vast majority of our analyses no outliers were identified and thus analyses were conducted using the complete sample set. Where the Grubb's test indicated an outlier, this generally resulted in the exclusion of a single sample from one group. The details of final sample numbers per group are stated in the figure/table legends. Since our primary research question was to determine the effect of genotype, we used *t*-tests to determine significant differences between Jz-*Igf2*UE and control groups within one sex. By performing separate analyses for males and females, we are able to control for the potential effect of sex. Statistical analyses were performed using Prism (GraphPad Software Inc.) and considered statistically significant when $P < 0.05$.

Results are expressed as mean $\pm$ S.E.M., with n =number of placentas or fetuses presented per group.

Results

Confirmation that *Igf2* has been successfully reduced in the placental Jz

We first sought to confirm that our genetic approach resulted in the specific reduction of *Igf2* in the placental Jz. The *Igf2* gene is expressed by the developing conceptus from the 2-cell stage (Stojanov *et al.* 1999) and the *Tpbpa* gene is expressed from D7 (Lescisin *et al.* 1988). Thus, *Tpbpa*Cre mediated loss of *Igf2* would be predicted to affect all Jz cell types from shortly after implantation at ~D5. Using qPCR on separated placental zones on D16 of pregnancy, we found that while *Igf2* expression was unaltered in the Lz, *Igf2* expression was reduced in the Jz of Jz-*Igf2*UE placentas of both female and male fetuses by 50% and 45%, respectively (Fig. 1A and B). Consistent with previous reports (Coan *et al.* 2006, Redline *et al.* 1993), *in situ* hybridisation demonstrated widespread expression of *Igf2* in the Lz and GC of control placentas, with low expression detected in the SpT and TGC (Fig. 1C and D). Expression of *Igf2* was maintained in the Lz of

Figure 1

Igf2 expression is significantly reduced in the Jz by qPCR and completely abolished in the Jz by *in situ* hybridisation in D16 mouse placentas with Jz-*Igf2*UE. (A and B) Expression of *Igf2* in isolated Jz and Lz samples relative to housekeeping geometric mean of *Hprt* and *Ywhaz* using qPCR in (A) males (control $n = 10$ and UE $n = 8$) and (B) females (control $n = 10$ and UE $n = 12$) across 8 litters. Values presented as mean $\pm$ S.E.M. with significance assessed by *t*-test ($P < 0.01 = **$). (C and D) *In situ* hybridization of *Igf2* in (C) males and (D) females. Black boxes represent the area magnified in the image. Black bar represents 1 mm. Red bar represents 100 μ m.

Table 1 The effect of Jz-Igf2UE on conceptus weight, placental volume and the proportion of cell types within the Jz and Db of D16 mouse placentas in males.

	Male	
	Control	Jz-Igf2UE
Fetal weight (mg)	409.0 ± 10.0	409.4 ± 10.2
Placental weight (mg)	114.4 ± 2.8	114.0 ± 2.3
Db volume (mm ³)	17.0 ± 0.9	15.5 ± 0.9
Jz volume (mm ³)	45.1 ± 3.0	44.5 ± 2.7
Lz volume (mm ³)	49.7 ± 1.0	50.5 ± 1.9
Jz_SpT volume (mm ³)	27.7 ± 1.8	26.4 ± 2.0
Jz_GC volume (mm ³)	15.6 ± 1.5	15.0 ± 1.5
Jz_TGC volume (mm ³)	2.3 ± 0.3	3.2 ± 0.5
Db_S volume (mm ³)	10.7 ± 0.8	9.6 ± 0.8
Db_GC volume (mm ³)	5.8 ± 0.6	5.1 ± 0.4
Db_V volume (mm ³)	0.9 ± 0.3	0.8 ± 0.2
Jz_GC/Jz_SpT	0.57 ± 0.05	0.60 ± 0.08
Db_GC/Jz_GC	0.39 ± 0.05	0.38 ± 0.06
Db_GC/(Jz_GC + Db_GC)	0.28 ± 0.02	0.27 ± 0.03
Average Jz_GC cell size (µm ²)	158.0 ± 11.0	149.3 ± 7.9

Weight data displayed for males: Control $n = 22$ and Jz-Igf2UE $n = 30$, across 14 litters, Control:Jz-Igf2UE = 42:58. Volume data: $n = 8$ per genotype, across 8 litters. Values presented as mean ± s.e.m. with significance assessed by t -test.
Db, Decidua; Db_GC, Db glycogen cell; Db_S, Db stroma; Db_V, Db vessel; Jz, Junctional zone; Jz_GC, Jz glycogen cells; Jz_SpT, Jz spongiotrophoblasts; Jz_TGC, Jz trophoblast giant cell; Lz, Labyrinth zone.

Table 2 The effect of Jz-Igf2UE on conceptus weight, placental volume and the proportion of cell types within the Jz and Db of D16 mouse placentas in females.

	Female	
	Control	Jz-Igf2UE
Fetal weight (mg)	386.4 ± 10.2	395.7 ± 7.1
Placental weight (mg)	112.0 ± 3.4	108.4 ± 3.1
Db volume (mm ³)	18.7 ± 1.4	18.8 ± 1.9
Jz volume (mm ³)	48.3 ± 2.2	41.7 ± 1.4 ^a
Lz volume (mm ³)	56.5 ± 3.0	53.2 ± 3.2
Jz_SpT volume (mm ³)	30.1 ± 1.5	25.9 ± 0.8 ^a
Jz_GC volume (mm ³)	14.8 ± 0.8	12.5 ± 0.5 ^a
Jz_TGC volume (mm ³)	2.9 ± 0.5	2.3 ± 0.3
Db_S volume (mm ³)	12.9 ± 1.2	11.7 ± 1.3
Db_GC volume (mm ³)	4.8 ± 0.3	6.1 ± 0.6
Db_V volume (mm ³)	0.9 ± 0.2	1.1 ± 0.2
Jz_GC/Jz_SpT	0.49 ± 0.02	0.49 ± 0.03
Db_GC/Jz_GC	0.33 ± 0.03	0.47 ± 0.06 ^a
Db_GC/(Jz_GC + Db_GC)	0.24 ± 0.01	0.31 ± 0.03 ^a
Average Jz_GC cell size (µm ²)	142.0 ± 2.7	149.3 ± 7.9

Weight data displayed for females: Control $n = 23$ and Jz-Igf2UE $n = 29$, across 14 litters, Control:Jz-Igf2UE = 44:56. Volume data: $n = 8$ per genotype, across 8 litters. Values presented as mean ± s.e.m. with significance assessed by t -test ($P < 0.05 = ^a$).
Db, Decidua; Db_GC, Db glycogen cell; Db_S, Db stroma; Db_V, Db vessel; Jz, Junctional zone; Jz_GC, Jz glycogen cells; Jz_SpT, Jz spongiotrophoblasts; Jz_TGC, Jz trophoblast giant cell; Lz, Labyrinth zone.

Jz-Igf2UE placentas, but *Igf2* could not be detected in the Jz of mutants of either sex (Fig. 1C and D). Thus, we were able to successfully ablate *Igf2* expression in the Jz.

Jz *Igf2* loss affects placental Jz structure but not fetal growth

Jz-Igf2UE did not affect fetal or placental weight of male or female conceptuses on D16 (Tables 1 and 2). Using stereology, gross placental structure and abundance of cell types in the Jz and Db were unaffected by Jz-Igf2UE in male fetuses (Table 1). However, Jz volume and the constituent volumes of SpT and GC in females was significantly decreased in response to Jz-Igf2UE (Table 2). There was no effect of Jz-Igf2UE on Lz or Db volume or abundance of Db cell types in the placentas of female fetuses (Table 2). Thus, Jz-Igf2UE affects the formation of the placental Jz in female, but not male placentas.

Jz *Igf2* loss does not affect Jz cell marker expression, glycogen storage or apoptosis levels

There were no significant changes in placental glycogen concentration or Jz GC size between control and Jz-Igf2UE placentas for either sex (Fig. 2A, B, C and D). Moreover, glycogen localisation using PAS staining to the placental Jz and Db GCs was not visibly different between control and Jz-Igf2UE placentas (Fig. 2E and F).

Expression of the Jz lineage markers, *Prl8a8* for SpT, *Gjb3* for GC and *Hand1* for TGC were measured in the Jz by qPCR to assess if they may be affected by Jz-Igf2UE (Fig. 3A and B). While there was no significant effect of Jz-Igf2UE on the expression of *Prl8a8* or *Hand1* in either sex *Gjb3* expression trended to be reduced in the placental Jz of male, but not female fetuses (Fig. 3A and B).

We next used *in situ* hybridisation for the Jz marker *Tpbpa* and the SpT marker *Prl8a8* to visually assess the integrity of the Db/Jz and Jz/Lz boundaries (Fig. 3C and D). The placental region boundaries appeared intact and there was no visual difference in *Tpbpa* or *Prl8a8* staining in response to Jz-Igf2UE, regardless of fetal sex. However, qualitatively, Jz interdigitation was less in females, but not males with Jz-Igf2UE compared to control (although females controls had more interdigitation than male controls). There was no difference in the levels of apoptosis in the Jz or Db of either males or females with Jz-Igf2UE as indicated by cleaved caspase-3 (Supplementary Figs 1C and 2). Thus, despite sexually disparate effects on Jz formation, Jz-Igf2UE does not alter the expression of endocrine cell

Figure 2

Jz-Igf2UE does not alter placental glycogen concentration or Jz GC size at D16 of mouse pregnancy. (A and C) Placental glycogen concentration in (A) males and (C) females with Jz-Igf2UE. $n = 7-8$ per sex and genotype, across 8 litters. (B and D) Size of Jz glycogen cells in (B) males and (D) females. $n = 4$ per sex and genotype, across 4 litters. (E and F) Pas stain of glycogen containing cells in (E) males and (F) females. Black boxes represent the area magnified in the image. Black bar represents 1 mm. Red bar represents 100 μm .

lineage marker genes, glycogen accumulation or apoptosis levels in placentas of either sex.

Jz *Igf2* loss affects the expression of the IGF2 signaling pathway in the placental Jz

To explore whether the fetal sex-dependent effects of Jz-Igf2UE on Jz morphology may be related to changes in the expression of signaling components downstream of IGF2, we quantified the mRNA expression of IGF2 receptors, *Igf1r*, *Igf2r* and *Insr*, as well as PI3K-AKT (*P85*, *P110 α* , *P110 β* , *Akt* and *Gsk3*) and RAS-MAPK-ERK (*H-ras*, *N-ras*, *K-ras*, *Mek1*, *Mek2*, *Erk1*, *Erk2* and *P38 α*) pathway genes in the placental Jz (Fig. 4). In the placenta of male fetuses, Jz-Igf2UE significantly reduced the expression of *Igf1r* by 45% and *Igf2r* by 16% in the Jz but had no effect on *Insr* (Fig. 4A). Conversely, Jz-Igf2UE increased *Insr* expression by 43% in the placental Jz of females without an effect on *Igf1r* or *Igf2r* (Fig. 4B). The expression of *p110 β* trended to be increased in females with Jz-Igf2UE, although, overall, there were no significant changes in the expression of PI3K-AKT pathway genes with Jz-Igf2UE in either fetal sex (Fig. 4C and D). However, there were sex-specific alterations to genes in the RAS-MAPK-ERK pathway with Jz-Igf2UE. Males with Jz-Igf2UE had significant decreases in the expression of *H-ras* (23%), *N-ras* (25%) and *K-ras* (30%) and showed trends for a

decrease in *Mek1* and *Mek2* (Fig. 4E). In females, Jz-Igf2UE significantly reduced the expression of *Mek1* by 16% and *P38 α* by 18% (Fig. 4F). Taken together, Jz-Igf2UE affects the expression of IGF2 signaling components by the placental Jz in a manner dependent on fetal sex.

Jz *Igf2* loss affects the expression of hormones by the placental Jz

To examine whether Jz-Igf2UE affects the placental capacity to produce hormones, we quantified the expression of selected *Prl* (*Prl3b1* and *Prl3d1*), *Psg* (*Psg21* and *Psg23*) and steroidogenic synthetic pathway genes (*Hmgcr*, *Stard1*, *Cyp11a1*, *3bhsd* and *Cyp17a1*) by qPCR (Fig. 5). In response to Jz-Igf2UE, the expression of *Cyp17a1* was significantly increased in the placental Jz of both males and females by 49% and 63%, respectively (Fig. 5A and B). Moreover, *Prl3b1* was significantly decreased in the placental Jz by 19% and 25% in females and males with Jz-Igf2UE, respectively (Fig. 5C and D). However, males, but not females, with Jz-Igf2UE also had reduced expression of *Hmgcr* and *Stard1* with values 23% and 29% lower than controls, respectively (Fig. 5A and B). There were non-significant trends for increased expression of *Psg21* in the placental Jz of female fetuses and decreased expression of *Prl2c2* in males with Jz-Igf2UE. Jz expression of *Cyp11a1*, *3bhsd*, *Prl3d1* and *Psg23* was not affected by

Figure 3

Expression and localisation of endocrine cell markers at D16. (A and B) Expression of endocrine cell markers relative to housekeeping geometric mean of *Hprt* and *Ywhaz* using qPCR in (A) males (control $n = 10$ and UE $n = 8$) and (B) females (control $n = 10$ and UE $n = 12$; except for *Gjb3* where UE $n = 11$ and *Hand1* UE $n = 10$), across 8 litters. Values presented as mean + s.e.m. with significance assessed by *t*-test. (C and D) *In situ* hybridization of *Tpbpa* and *Prl8a8* in mouse placentas in (C) males and (D) females. Black boxes represent the area magnified in the image. Black bar represents 1 mm. Red bar represents 100 μm .

Figure 4

Jz-Igf2UE alters the expression of IGF2 signaling factors but not downstream members of the PI3K-AKT pathway. Expression of (A and B) IGF2 signaling factors, (C and D) PI3K-AKT pathway genes and (E and F) RAS-MAPK-ERK pathway genes relative to housekeeping geometric mean of *Hprt* and *Ywhaz* with *Jz-Igf2UE* using qPCR in (A, C and E) males (control $n = 10$ and UE $n = 8$) and (B, D and F) females (control $n = 10$ and UE $n = 12$; except for *H-ras* where control $n = 9$), across 8 litters. Values presented as mean + s.e.m. with significance assessed by *t*-test ($P < 0.05 = *$, $P < 0.001 = ***$).

Figure 5

Jz-Igf2UE alters the expression of Jz hormones and steroidogenic pathway genes. Expression of (A and B) steroidogenic pathway regulatory genes and (C and D) selected mouse *Prl* and *Psg* genes relative to housekeeping geometric mean of *Hprt* and *Ywhaz* with *Jz-Igf2UE* using qPCR in (A and C) males (control $n = 10$ and UE $n = 8$; except for *Cyp17a1* where control $n = 9$) and (B and D) females (control $n = 10$ and UE $n = 12$; except for *Cyp11a1* and *Cyp17a1* control $n = 9$ and *Stard1*, *Cyp11a1*, *3bhsd*, *Prl3b1* and *Prl3d1* UE $n = 11$), across 8 litters. Values presented as mean $\pm$ s.e.m. with significance assessed by *t*-test ($P < 0.05 = *$, $P < 0.01 = **$).

Jz-Igf2UE in males or females (Fig. 5). Thus, *Jz-Igf2UE* affects the expression of hormone-related genes by the placental Jz in a manner that partly depends on sex of the fetus.

Discussion

To date, our understanding of the role of IGF2 in the mouse placenta has been limited to global or Lz specific manipulations of *Igf2*. In this study, we generated a novel model of Jz-specific loss of *Igf2* to examine its significance in regulating mouse placental endocrine function. The specificity of the knockout was validated using *in situ* hybridisation and qPCR analysis of *Igf2* expression. In the current study, we found that *Jz-Igf2UE* compromised the formation of SpT and GC in the placental Jz and the capacity for steroid and placental lactogen hormone production. These changes were related to alterations in the expression of receptors and signaling pathways that are responsive to IGF2 and depended on the sex of the fetus. Taken together, these data show that Jz-derived *Igf2* regulates placental endocrine capacity in the mouse in a manner that is determined, in part, by fetal sex.

Global loss of *Igf2* in mice led to fetoplacental growth restriction during the last two-thirds of gestation (Baker *et al.* 1993, DeChiara *et al.* 1990, 1991). Moreover, deletion of the Lz specific *Igf2P0* transcript also led to fetoplacental growth restriction (Constância *et al.* 2002). However, in this study, *Jz-Igf2UE* had no significant effect on fetal or placental weights compared to controls. This suggests that Jz *Igf2* is not directly responsible for regulating fetal or placental growth up until day 16 of pregnancy in a litter with fetuses of different genotypes. Indeed, Lz *Igf2* may be more essential for placental growth up to this stage of pregnancy, as the *Igf2P0* knockout placentas displayed the same degree of growth restriction as global *Igf2* knockouts

until day 14 (Constância *et al.* 2005). To minimise possible effects of variations in the maternal environment on fetoplacental phenotype due to *Jz-Igf2UE*, data in this study were collected from litters of mixed genotype. However, the presence of a mutant placenta that has perturbed functional capacity can influence the phenotype of genetically wild-type littermates (Tunster *et al.* 2016a, López-Tello *et al.* 2019). Thus, there could be changes in the fetoplacental phenotype of both the control and *Jz-Igf2UE* that we were unable to identify due to the shared gestational environment. Future work should compare fetoplacental phenotype between litters that are entirely wild-type and entirely *Jz-Igf2UE* mutant.

While there was no reduction in placental weight, Jz volume was significantly reduced by *Jz-Igf2UE* for female fetuses. This was partly due to decreased Jz GC and Jz SpT volumes and was associated with qualitatively less Jz interdigitation in females, but not in males with *Jz-Igf2UE* compared to control. We do not know the biological significance of altered Jz interdigitation in *Igf2UE* female fetuses. However, previous studies have shown that the integrity of the Jz/Lz boundary is susceptible to genetic manipulation (Tunster *et al.* 2010, 2016b, Naruse *et al.* 2014, Hasan *et al.* 2015, Camm *et al.* 2018), our data would now suggest this may occur in a manner dependent on fetal sex. Nonetheless, the reduced GC volume found in *Igf2UE* female fetuses correlates with previous research showing that constitutive loss of *Igf2* leads to an 80% reduction in the number of glycogen containing cells in the placenta (Lopez *et al.* 1996). However, the GC volume reduction seen in the female *Jz-Igf2UE* placentas is less severe (16%). Deletion of *Igf2P0* (Lz specific IGF2) also results in reduced placental GC at a higher level than observed with *Jz-Igf2UE* (Sferruzzi-Perri *et al.* 2011b). This suggests that IGF2 from the Lz may be more important in the formation or maintenance of GC in the Jz. Alternatively, as GC normally decline in abundance from D16.5 to term

(Coan *et al.* 2006), the reduced Jz GC volume in females could be due to an early activation or accelerated loss of GC with Jz-*Igf2*UE.

Despite previous work showing that IGF2 can exert an anti-apoptotic function (Sferruzzi-Perri *et al.* 2017), we found no difference in the levels of the apoptotic marker, caspase-3 in the placental Jz in response to Jz-*Igf2*UE. The decrease in Jz GC volume but lack of a change in Jz apoptosis, GC size or glycogen content in female placentas suggests that Jz-*Igf2*UE leads to fewer GCs with normal glycogen storage capacity compared to the control female placentas. This finding differs from previous work in ubiquitous null mice showing that *Igf2* increases the activity of the glycogen synthesis pathway in the placenta (Lopez *et al.* 1996) and other tissues, like the liver (Lopez *et al.* 1999, Liang *et al.* 2010). The PI3K-AKT pathway promotes glucose uptake and glycogen synthesis downstream of ligands like IGF2 (Forbes *et al.* 2008, Huang *et al.* 2018) and disrupted PI3K-AKT signaling leads to reduced glycogen accumulation in the placenta of mice (Dong *et al.* 2012, Sferruzzi-Perri *et al.* 2016). In females, but not males, Jz-*Igf2*UE significantly increased the expression of *Insr* in the placental Jz. There was also a trend for increased *p110 β* , suggesting enhanced capacity for PI3K-AKT signaling in females and not males with Jz-*Igf2*UE. INSR has almost as high an affinity for IGF2 as its natural ligand, insulin. INSR also has a much lower affinity for IGF1, which instead has the highest affinity for IGF1R (Germain-Lee *et al.* 1992, Blanquart *et al.* 2008). Our data suggest that females may respond to Jz *Igf2* loss by increasing the ability of IGF2 to bind INSR to stimulate PI3K-AKT signaling pathway activation and maintain placental glycogen synthesis. In males, Jz-*Igf2*UE significantly decreased *Igf2r* expression and did not affect PI3K-AKT pathway expression in the placental Jz. Unlike IGF1R, IGF2R has no tyrosine kinase activity and primarily acts as a clearance receptor by targeting IGF2 for lysosomal degradation (Frago *et al.* 2016). Therefore, our findings indicate that the placenta of males responded to Jz-*Igf2*UE by decreasing the degradation of IGF2, which, in turn, may have preserved PI3K-AKT signaling and glycogen synthesis. Taking our findings from both sexes together, our data demonstrate that the placenta may compensate for *Igf2* loss in the Jz by increasing IGF2 signaling capacity via two different mechanisms, increased sensitivity and increased ligand abundance in female and male fetuses, respectively.

IGF2 signals via the MAPK pathway to promote proliferation (Forbes *et al.* 2008). Therefore, it is perhaps not surprising that components of the MAPK signaling

pathway were downregulated by Jz-*Igf2*UE in both the placental Jz of male and female fetuses. However, while the level of *Igf2* loss was comparable in both fetal sexes, the expression of specific components in the MAPK signaling pathway in the placental Jz differed between females and males. In males, early components of the MAPK pathway, *H-ras*, *N-ras* and *K-ras*, were significantly reduced and there were trends for a decrease seen with *Mek1* and *Mek2*, which are further down the pathway, in response to Jz-*Igf2*UE. In contrast, in females, only *Mek1* and *P38 α* expression were decreased by Jz-*Igf2*UE. The greater reduction in MAPK signaling in males with Jz-*Igf2*UE is consistent with the diminished *Igf1r* expression seen in male fetuses, but not females; as IGF1R activates the RAS-MAPK-ERK (Forbes *et al.* 2008). However, the formation of the Jz and its constituent volume of GCs were compromised by Jz-*Igf2*UE in female and not male fetuses. There are also sexually disparate differences in IGF1R expression and MAPK signaling activity in the rodent placenta in response to maternal manipulations including exercise, dexamethasone treatment, inhalation hypoxia and alcohol intake (Cuffe *et al.* 2011, 2014, Gårdebjer *et al.* 2014, Briffa *et al.* 2017, Mangwiro *et al.* 2018). Maternal alcohol consumption in rats and dexamethasone treatment in mice also disrupts the formation of the Jz and GC in placenta specifically of female fetuses (Cuffe *et al.* 2011, Gårdebjer *et al.* 2014).

In the current study, disparities in the placenta Jz phenotype with Jz-*Igf2*UE between the fetal sexes are likely related to the specific components of the MAPK pathway that are altered. For instance *Mek1* and particularly *P38 α* loss in mice are associated with defects in Jz formation in mice (Giroux *et al.* 1999, Mudgett *et al.* 2000, Li *et al.* 2003, Abell *et al.* 2009). In the present study, *Igf1r* expression by the Jz was unaltered by Jz-*Igf2*UE in female fetuses. *Igf2r* was also unchanged and *Insr* adaptively increased by Jz-*Igf2*UE in females. Previous work has shown that loss of the *Insr* and *Igf1r* in mice does not affect placental development, including the formation of the Jz or GCs (Esquiliano *et al.* 2009). Collectively our data and that of others support the notion for the existence of an unknown receptor that mediates the effects of IGF2 on placental formation (XR_p) (Baker *et al.* 1993, Esquiliano *et al.* 2009). However, our data additionally suggests that XR_p may function or is differentially responsive to challenges, like reduced *Igf2* availability in the placenta of males and females. INSR and IGF1R hybrids and INSR isoforms have been reported to be expressed by the human placenta (Soos *et al.* 1993). Future work should explore the contribution of these

hybrid receptors to IGF2-mediated effects in the placenta, including the Jz in mice.

The expression of *Prl3b1* was reduced and expression of *Cyp17a1* was increased in males and females by Jz-Igf2UE. *Prl3b1*, which encodes placental lactogen 2 (PL2), is expressed by SpT and several TGC lineages (Simmons *et al.* 2008). Previous work has also shown a positive correlation of *Prl3b1* and *Igf2* expression/signaling by the placenta in rodents (Ain *et al.* 2005, Ishida *et al.* 2007, Sferruzzi-Perri *et al.* 2016, Napso *et al.* 2019). PL2 maintains production of progesterone by the corpus luteum in culture (Thordarson *et al.* 1997) and stimulates production of insulin by neonatal mouse cultured pancreatic islets (Brelje *et al.* 1993). *Cyp17a1* encodes a key steroidogenic enzyme that promotes the production of estrogens (Miller 2002). Estrogen is important for increasing maternal insulin sensitivity and maintaining glucose homeostasis during pregnancy (Brănișteanu & Mathieu 2003). IGF2 has been shown to increase *Cyp17a1* expression in human adult (L'Allemand *et al.* 1996) and fetal adrenocortical cells (Mesiano & Jaffe 1993) and stimulates estrogen production in bovine granulosa cells (Spicer & Aad 2007). Together, this suggests that IGF2 may exert tissue-specific effects on estrogen production or that the upregulated placental Jz *Cyp17a1* expression may be a secondary effect of the Jz-Igf2UE. In the placentas of male fetuses, Jz-Igf2UE also trended to reduce the expression of *Prl2c2*, which encodes proliferin, and significantly decreased the expression of additional steroidogenic pathway genes, *Hmgcr* and *Stard1*. Earlier work has demonstrated that IGF2 and activation of the RAS-MAPK-ERK pathway increased *Stard1* expression and steroidogenesis in adrenocortical and prostate cancer cells (Gyles *et al.* 2001, Lubik *et al.* 2013). The greater impact on steroidogenic gene expression in the placental Jz by Jz-Igf2UE in males may therefore be related to the observed diminished expression of early components of the RAS-MAPK-ERK pathway. Regardless of the mechanisms involved, future work is required to determine whether physiological adaptations in the mother may be hindered due to altered placental steroidogenesis and *Prl3b1* expression when she is carrying litters containing Jz-Igf2UE mutants.

This study has illustrated that the resultant placental endocrine phenotype exhibited by males and females differs in response to Jz-Igf2UE. However, the mechanisms underlying the sexually discrepant findings are largely unknown. In murine development, sexually dimorphic alterations in epigenetic regulation have been observed as early as the eight-cell stage (Lowe *et al.* 2015).

Additionally, while the paternal X chromosome is selectively inactivated in mice, numerous placental genes can escape this, including *Slc38a5*, which encodes an amino acid transporter (Finn *et al.* 2014). Therefore, increased dosage of X-inactivation escaping genes in females may contribute to sex-specific differences. Conversely, the Y chromosome contains novel coding genes (Gubbay *et al.* 1990, Yamauchi *et al.* 2014), which may influence placenta function and account for the observed sexual dimorphism. Furthermore, hormone production by the fetal gonads or adrenal gland, which are active from D10–13.5 and vary between male and females (Kalisch-Smith *et al.* 2017), may also aid in the sexually distinct changes in placental phenotype observed.

Other studies have also shown sexually dimorphic responses to a pregnancy insult (Clifton 2010). For instance, there was a greater alteration in placental gene expression in females vs males in response to maternal dietary perturbations in mice (Mao *et al.* 2010). There are also variations in the number and types of genes involved in specific functions like nutrient metabolism in response to a maternal high-fat diet in the placenta of female and male fetuses (Gabory *et al.* 2012). Females, but not males, also displayed a reduction in placental mass and Jz weight in response to maternal dexamethasone treatment in mice (Cuffe *et al.* 2011). Female rodents also more readily displayed adaptations in placental morphology and gene expression in response to maternal hypoxia (Cuffe *et al.* 2014) and advanced age (Napso *et al.* 2019). Overall, the reason why a greater phenotypic response to Jz-Igf2UE is observed in female placentas remains unclear. However, it is clear that this is not a unique phenomenon and that sexually dimorphic responses in the placenta need to continue to be addressed in the future.

It is important to note that the expression of placental hormones and signaling pathway genes in the current study was assessed only at the RNA level, with no assessment of protein levels or activity. While future work should prioritise measuring the levels of proteins, including the phosphorylation status of those within the PI3K-AKT and MAPK pathways in the placenta (Sferruzzi-Perri *et al.* 2011b, Higgins *et al.* 2016), currently there are no reliable protein assays to assess individual murine PRL or PSG subtypes in the placenta or maternal circulation. Moreover, as pregnant dams used in the current study carried litters of mixed genotype, the effect of Jz-Igf2UE on placental hormone output and, hence, maternal circulating abundance will be diluted by the contribution of hormones coming from the control placentas she is also carrying. Subject to the availability of commercially

reliable assays, maternal serum hormone concentrations could be assessed by generating litters comprised entirely of one genotype in future work. Alternatively, the secretome of individual placentas from Jz-Igf2UE mixed genotype litters could be explored *in vitro*.

In conclusion, Jz-Igf2UE alters the cellular composition and hormone expression of the Jz in the mouse placenta. However, the nature of these changes was dependent on fetal sex. Despite changes in placental endocrine phenotype with Jz-Igf2UE, there was no impact on fetal or placental weight in a litter of mixed genotype at day 16. Future studies are required to determine whether altered placental endocrine phenotype may be associated with changes in maternal physiology and in the postnatal health of the offspring. Additionally, it is yet to be determined what impact Jz-specific *Igf2* overexpression has on the endocrine phenotype of the mouse placenta. Our findings may be important in the context of human pregnancy, as dysregulated expression of *Igf2*, its signaling pathway and placental hormones have been reported in human placentas in women with abnormal fetal growth and gestational diabetes (Laviola *et al.* 2005, Le *et al.* 2013, Su *et al.* 2016, Liao *et al.* 2017, Ngala *et al.* 2017).

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0128>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data availability

The datasets generated and analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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