Loss of imprinting of the \textit{Igf2-H19} ICR1 enhances placental endocrine capacity via sex-specific alterations in signalling pathways in the mouse.

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Short title
ICR1 and the endocrine placenta

Keywords
Insulin-like Growth Factor; Placenta; Hormones; Endocrine Cells; Trophoblast; Imprinted Gene

Word count
6658 words

Summary statement
Imprinting at \textit{Igf2-H19} ICR1 regulates endocrine cell formation and function via sexually-dimorphic changes in PI3K-AKT and MAPK signalling in the mouse.
Abstract

Imprinting control region (ICR1) controls the expression of the *Igf2* and *H19* genes in a parent-of-origin specific manner. Appropriate expression of the *Igf2-H19* locus is fundamental for normal fetal development, yet the importance of ICR1 in the placental production of hormones that promote maternal nutrient allocation to the fetus is unknown. To address this, we used a novel mouse model to selectively delete ICR1 in the endocrine junctional zone (Jz) of the mouse placenta (Jz-ΔICR1). The Jz-ΔICR1 mice exhibit increased *Igf2* and decreased *H19* expression specifically in the Jz. This was accompanied by an expansion of Jz endocrine cell types due to enhanced rates of proliferation and increased expression of pregnancy-specific glycoprotein 23 in the placenta of both fetal sexes. However, changes in the endocrine phenotype of the placenta were related sexually-dimorphic alterations to the abundance of IGF2 receptors and downstream signalling pathways (PI3K-AKT and MAPK). There was no effect of Jz-ΔICR1 on the expression of targets of the *H19* embedded miR-675 or on fetal weight. Our results demonstrate that ICR1 controls placental endocrine capacity via sex-dependant changes in signalling.
Introduction

In mammals, a subset of autosomal genes exhibit monoallelic (Ferguson-Smith and Surani, 2001) or preferential expression of one allele (Khatib, 2007; Schulz et al., 2006) in a parent-of-origin dependent manner. The expression of such imprinted genes is regulated by epigenetic mechanisms, such as DNA methylation, chromatin remodelling, and reciprocal expression of long non-coding RNA (Ferguson-Smith, 2011). To date, 260 imprinted genes have been identified in mice and 228 in humans, with the imprinting status of 63 genes conserved in both species (Tucci et al., 2019). Rather than being dispersed throughout the genome, imprinted genes typically co-localise in clusters, or imprinted domains, that are co-ordinately regulated by an imprinting control region (ICR) or imprinting centre (IC) (Cattanach and Kirk, 1985; Verona et al., 2003).

The first evidence of genomic imprinting came from pioneering nuclear transplantation experiments undertaken in the 1980s. The developmental failure of conceptuses carrying two paternal genomes (androgenetic) or two maternal genomes (parthenogenetic or gynogenetic) established the absolute requirement of both parental genomes for successful feto-placental development (Barton et al., 1984; Mann and Lovell-Badge, 1984; McGrath and Solter, 1984; Surani et al., 1984). Whilst several theories exist that attempt to explain the evolutionary origins of imprinting (reviewed in Edwards et al., 2019), the most prominent is the parental conflict hypothesis (Moore and Haig, 1991). Essential for mammalian viviparous reproduction is the substantial investment of maternal resources, such as the provision of nutrients from mother to fetus throughout gestation. The parental conflict hypothesis theorises that whilst the father (acting through the paternal genome) is primarily interested in achieving maximal offspring growth, the mother (acting through the maternal genome) must balance supporting growth of the offspring with need for nutrients to sustain her own health, and to support future offspring. Consequently, paternally expressed imprinted genes would be expected to promote fetal growth, whilst maternally expressed imprinted genes would be expected to limit growth (Moore and Haig, 1991).

The placenta functions as the interface between mother and fetus during pregnancy, and it is therefore unsurprising that many imprinted genes exert their influence upon fetal growth by regulating growth, development, and function of this vital organ (Tunster et al., 2013). Indeed, placentation and genomic imprinting are thought to have co-evolved ~168 million years ago (reviewed in Kaneko-Ishino and Ishino, 2019). Arguably the primary role of the placenta is to mediate nutrient and oxygen transfer to fetus (Burton and Fowden, 2015;
Sferruzzi-Perri and Camm, 2016). However, the placenta is also a major endocrine organ, producing an abundance of hormones and signalling factors that act systemically to adapt maternal physiology, metabolism and behaviour to support fetal growth and sustain pregnancy (Napso et al., 2018). We recently reported that the placental secretome comprises in excess of 300 proteins, including known factors such as steroid hormones, prolactin/placental lactogens and pregnancy specific glycoproteins (PSGs), as well as novel secreted placental proteins (Napso et al., 2020). Secreted placental proteins such as prolactins (PRLs) and steroidogenic hormones act systemically to regulate maternal insulin production, insulin sensitivity/resistance and glucose metabolism (Ahmed-Sorour and Bailey, 1980; Brelje et al., 2004; Huang et al., 2009; Jarrett et al., 1984; Petres and Sferruzzi-Perri, 2021; Sferruzzi-Perri et al., 2020; Wada et al., 2010). Moreover, PSGs can also act locally to promote immune-modulation and angiogenesis to support fetal development (Blois et al., 2012; Snyder et al., 2001). An imbalance in the allocation of nutrients between the mother and fetus has been linked to abnormal in utero development and lifelong health complications for offspring (Camm et al 2018; Fowden et al., 2006; Gluckman et al., 2008; Sferruzzi-Perri et al., 2013a).

In humans, both transport and endocrine functions are performed by syncytiotrophoblast cells of the placenta (Dearden and Ockleford, 1983), whereas in the mouse, these functions are performed by the structurally distinct labyrinth zone (Lz) and junctional zone (Jz), respectively. The Jz primarily comprises three trophoblast lineages; the spongiotrophoblast (SpT), glycogen cells (GC), and trophoblast giant cells (TGC). These Jz cell types derive from a common Tpbpa-positive precursor (Lescisin et al., 1988; Simmons et al., 2007) and have the capacity to produce a variety of hormones, including members of the PRL family, steroidogenic hormones and PSGs (Lavoie and King, 2009; McLellan et al., 2005; Simmons et al., 2008). Additionally, GC accumulate stores of glycogen and are considered to be analogous to the extravillous cytotrophoblast cells of the human placenta (Georgiades et al., 2002; Wislocki and Bennett, 1943).

Numerous mouse models exist that establish the vital role for imprinted genes in regulating placental nutrient transport (Angiolini et al., 2006; Coan et al., 2005). Perhaps key amongst these is the paternally expressed Igf2, which encodes for Insulin-like Growth Factor 2 (IGF2) and is highly expressed in placental and fetal tissues of humans (Han et al., 1987, 1988) and mice (DeChiara et al., 1991; Sferruzzi-Perri, 2018). Consistent with the parental conflict hypothesis, paternal inheritance of an Igf2-null allele restricts fetal-placental growth (Baker et
al., 1993; DeChiara et al., 1990, 1991). This fetal growth restriction (FGR) can be attributed, at least in part, to a placental defect, with placenta-specific loss of \textit{Igf2} also restricting fetoplacental growth (Constância et al., 2002).

\textbf{IGF2} is a potent promoter of cellular proliferation and differentiation, acting through the insulin receptor (INSR) or type-1 IGF receptor (IGF1R) to activate the RAS-MAPK-ERK or PI3K-AKT signalling pathways (reviewed in Czech, 1989; Forbes and Westwood, 2008; Jones and Clemmens, 1995; Sferruzzi-Perri et al., 2017; Siddle, 2011). Binding of IGF2 to INSR and activation of the PI3K-AKT signalling pathway also regulates glucose uptake and glycogen synthesis (Cross et al., 1995; Forbes and Westwood, 2008; Huang et al., 2018; Sferruzzi-Perri et al., 2016). IGF2 is thought to be cleared from the circulation via targeted lysosomal degradation following binding to the type-2 IGF receptor (IGF2R) (Czech, 1989; Lau et al., 1994; Morgan et al., 1987), which is encoded by the \textit{Igf2r} gene that in mice is also imprinted, with expression derived from the maternally inherited allele (Barlow et al., 1991).

\textit{Igf2} localises to the ICR1 imprinted domain on chromosome 7 in mice. This domain consists of the paternally expressed \textit{Ins2}, \textit{Igf2} and \textit{Igf2as}, the maternally expressed long non-coding \textit{H19} RNA and the microRNAs \textit{mir-675} and \textit{mir-483}. \textit{Igf2} and \textit{H19} share a common enhancer element, with imprinting mediated through paternal methylation of the differentially methylated region (DMR) ICR1 located ~4 kb upstream of \textit{H19} (Ferguson-Smith et al., 1993; Leighton et al., 1995b; Thorvaldsen et al., 1998; Tremblay et al., 1997). ICR1 contains recognition motifs for the zinc-finger DNA-binding protein CTCF, which blocks the interaction with enhancer elements (Bell et al., 1999; Szabo et al., 2000). Binding of CTCF to the hypomethylated maternal ICR1 prevents interaction of the \textit{Igf2} promoter with downstream enhancers, inactivating the maternal \textit{Igf2} allele (Bell and Felsenfeld, 2000; Kanduri et al., 2000; Szabo et al., 2000) and instead promotes the transcription of \textit{H19} (Engel et al., 2006; Schoenherr et al., 2003). In contrast, CTCF is unable to bind to the methylated paternal allele, allowing interaction of the \textit{Igf2} promoter with downstream enhancer elements and enabling its transcription. The absence of CTCF renders the paternal \textit{H19} allele inactive.

Maternal inheritance of a 13 kb deletion spanning \textit{H19} and ICR1 (\textit{H19}^{13}) results in fetoplacental overgrowth in mice (Leighton et al., 1995a). However, normalisation of fetal growth in mice inheriting the \textit{H19}^{13} allele maternally and an \textit{Igf2} null allele paternally isolates this fetal over-growth to over-expression of \textit{Igf2} rather than loss of function of \textit{H19} (Leighton et al., 1993).
Indeed, maternal inheritance of a ~1.6 kb deletion spanning ICR1 results in reactivation of the maternal $Igf2$ allele and overgrowth of neonates relative to control littermates (Thorvaldsen et al., 1998). Much of the subsequent investigation of $Igf2$ function in the placenta has focused on its role in regulating nutrient transport function (Angiolini et al., 2011; Coan et al., 2008; Constância et al., 2005; Sibley et al., 2004). However, both global and placenta-specific loss of $Igf2$ also restrict Jz size alongside impacting Lz size and function (Coan et al., 2008; Sferruzzi-Perri et al., 2011). Furthermore, $Igf2$ is highly expressed by GC (Redline et al., 1993), with constitutive loss of $Igf2$ resulting in reduced GC abundance and placental glycogen stores (Lopez et al., 1996), whilst ubiquitous maternal inheritance of the $H19^{13}$ allele results in an expansion of the GC population and increased placental glycogen content (Esquiliano et al., 2009).

Recent mouse studies demonstrate an emerging role for imprinted genes in regulating placental endocrine capacity (reviewed in John, 2013, 2017). For instance, over-expression of the maternally expressed imprinted genes $Phlda2$ and $Ascl2$ result in a reduction in Jz size (Tunster et al., 2015, 2016), suggesting that imprinting (paternal silencing) of these genes enhances placental endocrine capacity. Conversely, loss of expression of the paternally expressed $Peg3$ also restricts Jz size (Tunster et al., 2018), suggesting that imprinting (maternal silencing) of $Peg3$ would act to restrict placental endocrine capacity. We recently reported that Jz-specific loss of $Igf2$ restricts placental endocrine capacity in a sexually-dimorphic manner (Aykroyd et al., 2020). We therefore hypothesised that the acquisition of imprinting of the ICR1 domain modulates placental endocrine capacity. Utilising a unique genetic model in which ICR1 is specifically deleted in cells of the placental Jz (Jz-$\Delta$ICR1), we sought to investigate the role of ICR1 imprinting in modulating placental endocrine function.
**Results**

**Validation of Jz specific Igf2-H19 imprinted gene dysregulation with Jz-ΔICR1**

Homozygous *TpbpaCre* males (Simmons et al., 2007) were mated to heterozygous ICR floxed females (LoxP sites surrounding the ICR; termed ICR1Flox; Srivastava et al., 2000) for a conditional deletion of the *Igf2* and *H19* ICR in the placental Jz (Fig. 1). This generated litters consisting of fetuses with control and Jz-ΔICR1 placentae (Fig. 1B) and using qPCR of isolated Jz obtained on gestational day (D) 16, we verified that Jz expression of *Igf2* was increased by 30% in males and 25% in females, whilst *H19* decreased by 36% in males and 39% in females (Fig. 2A). In contrast, expression of *Igf2* and *H19* was unaltered in the Lz of Jz-ΔICR1 placentae. To ensure that Jz-ΔICR1 did not result in ectopic expression of *Igf2* or *H19*, we assessed their spatial expression by *in situ* hybridisation. Consistent with previous work (Aykroyd et al., 2020; Coan et al., 2006; Redline et al., 1993) we observed high levels of *Igf2* expression in the Lz and GC, with lower levels of expression in SpT and TGC of both control and Jz-ΔICR1 placentae on D16 (Fig. 2B). In control and Jz-ΔICR1 placentae, *H19* was also widely expressed in the Lz, although expression was restricted to the GC in the Jz (Fig. 2C). Negative controls for the *in situ* hybridisations are shown in Fig. S1A,B.

**Jz-ΔICR1 affects placental structure, through enhanced proliferation of endocrine cells, but does not affect fetal growth**

There was no difference in fetal weight (Fig. 3A) or placental weight (Fig. 3B) in response to Jz-ΔICR1. Regardless of genotype, placentae of males were heavier than females (Fig. 3B). Jz-ΔICR1 increased Jz volume by 20% in males and 43% in females. There was an overall effect of Jz-ΔICR1 to decrease Lz and Db volume, with a significant effect of lower Db volume for male conceptuses only (Fig. 3C). Jz volume was lower in placentae of females compared to males, an effect significant in control but not Jz-ΔICR1 conceptuses (Fig. 3C).

Further stereological analysis of the placenta revealed that increased Jz volume with Jz-ΔICR1 was attributable to increased volume of SpT (+33%), GC (+51%) and TGCs (+96%) in females and increased volume of SpT (+22%) in males (Fig. 3D). The total volume of SpT in both genotypes and total volume of GC in controls was less in female placentas compared to males. The distribution and average cell size of SpT cells in the Jz was unaffected by
genotype (Fig. 3E and F), however, the average number of SpT cells was increased by 44% in females and 34% in males in response to Jz-ΔICR1 (Fig. 3G). The average number of SpT cells in Jz-ΔICR1 placentas was less in females compared to males (Fig. 3G). The distribution and average cell size of Jz GC cells was also unaffected by genotype (Fig. 3H and I), although there was a greater percentage of GC that were within the 100 µm² size range in control females versus control males (Fig. 3H). There was also a 63% increase in the average number of Jz GC cells in female placentas with Jz-ΔICR1, but no significant effect observed in males (Fig. 3J). There was an interaction between genotype and sex in determining the total volume of decidual stroma (Db_S); whereby Db_S volume was reduced by Jz-ΔICR1 in male, but not female conceptuses, and Db_S volume was greater in Jz-ΔICR1 females compared to Jz-ΔICR1 males (Fig. 3K). There was no effect of genotype or sex on the volume of GC and vessels in the decidua.

As indicated by Ki67 staining, there was an overall >3-fold increase in cell proliferation in the Jz of Jz-ΔICR1 conceptuses, an effect significant in all three Jz cell types (Fig. 4B). In contrast, as determined by cleaved caspase-3 staining, there was no effect of Jz-ΔICR1 on the number of cells undergoing apoptosis in the Jz (Fig. 4D). There was no effect of fetal sex on Jz cell proliferation or apoptosis. Representative negative control images can be found in Fig S1C,D.

**Jz-ΔICR1 did not affect the expression of Jz cell markers but increased total placental glycogen storage in females**

Whilst total volumes of the three Jz lineages were increased, expression of the SpT marker Prl8a8, the GC markers Gjb3 and Pcdh12, and the TGC marker Hand1 were all unaffected by Jz-ΔICR1 at the cellular level regardless of fetal sex (Fig. 5A). However, expression of Gjb3, Pcdh12 and Hand1 was lower in the placental Jz from females compared to males, and in the case of Gjb3 and Pcdh12, pairwise comparisons revealed this was significant for controls only (Fig. 5A). We next investigated whether the increased GC volume in Jz-ΔICR1 placentas impacted placental glycogen metabolism. Jz-ΔICR1 did not affect the expression of the glucose transporter Slc2a1 or key glycogen synthesis pathway genes in the placental Jz (Fig. 5B). However, overall, Gys1 and Gbe1 were more highly expressed in the placentae of males compared to females (Fig. 5B). Placental glycogen concentration was also not altered by Jz-ΔICR1 (Fig. 5C). However, total placental glycogen was increased by Jz-ΔICR1, an effect that was significant in females (+34%) when data were separated by sex (Fig. 5D). Visualisation of GC by PAS staining did not identify any overt differences in the
spatial localisation of GC in the placenta in response to Jz-ΔICR1 (Fig. 5E). We further assessed the integrity of the Jz/Lz and Db/Jz boundaries by in situ hybridisation for the Jz marker Tpbpa and the SpT marker Prl8a8 and qualitative assessment revealed no overt differences between control and Jz-ΔICR1 placentas (Fig. 5F,G).

**Jz-ΔICR1 results in increased expression of the Jz hormone Psg23**

We next investigated whether placental endocrine function was affected by Jz-ΔICR1. The expression of the steroidogenic pathway genes Hmgcr, Stard1, Cyp11a1, Hsd3b1 and Cyp17a1 were unaltered by Jz-ΔICR1, although Stard1 was expressed at a lower level in the placental Jz of females compared with males (Fig. 6A). Similarly, expression of members of the Prl gene family Prl2c2, Prl3b1, Prl3d1, Prl6a1 and Prl7b1 and the angiogenic regulators Flt1 and Vegfa were unaffected by Jz-ΔICR1, although expression of Prl2c2 and Prl3d1 by the placental Jz was lower in females compared to males (Fig. 6B and C). Whilst expression of Psg21 was unaffected by Jz-ΔICR1, we observed a >2-fold increase in expression of Psg23 in the Jz from both males (+2.3-fold) and females (+2.2-fold) (Fig. 6C). Overall, expression of Psg23 was lower in placental Jz of females compared to males (Fig. 6C). In situ hybridisation revealed that in control placentas, Psg23 was localised to the Jz, with high levels of expression in the SpT and weak expression within GC. The spatial localisation of Psg23 was maintained in Jz-ΔICR1 placentas, although staining intensity was notably greater when compared to controls (Fig. 6D).

**Jz-ΔICR1 alters the protein expression of IGF2 signalling factors**

To inform on the mechanism through which loss of imprinting of the ICR1 domain (via Jz deletion of ICR1) regulates Jz development, we quantified the abundance of IGF2 receptors (IGF1R, IGF2R and INSR) and downstream members of the PI3K-AKT and MAPK pathways (PI3K subunits P85, P110α, P110β, as well as phosphorylated (p) and total (T-) AKT, GSK3, P38 and MAPK 42/44) using western blotting (Fig. 7). In males, p/T-AKT (phosphorylated to total AKT), T-GSK3, pP38, p/T-P38 (phosphorylated to total P38) and pMAPK were significantly increased by Jz-ΔICR1, with a trend for an increase in P110β (p=0.07) (Fig. 7A,C). However, the levels of IGF1R, T-AKT and p/T-GSK3 (phosphorylated to total GSK3) were significantly decreased in the Jz of males with Jz-ΔICR1. Conversely, in females, there was a significant increase in INSR, P85, pAKT, T-AKT, with a trend for an increase in IGF2R (p=0.08) in the Jz in response to Jz-ΔICR1 (Fig. 7B,D). Females with Jz-ΔICR1 also had a significant decrease in the level of T-GSK3 and a trend for a decrease in T-MAPK (p=0.06),
compared to controls. Changes in protein abundance with Jz-ΔICR1 were not associated with corresponding changes in gene expression as assessed by qPCR in either the placental Jz of male or female fetuses (Fig. S2). Although there was a significant effect of fetal sex on Jz expression of Insr, Gsk3, N-ras and Mek1 (Fig. S2).

Other contributing mechanisms

Cre-mediated ablation of the transcription factor Tfap2c leads to a reduction in Jz size, with increased expression of H19 and decreased expression of Psg23 in isolated Jz (Sharma et al., 2016). Thus, we examined whether expression of Tfap2c was altered in response to Jz-ΔICR1 and could contribute to the placental Jz phenotype observed. However, there was no significant effect of Jz-ΔICR1 on Jz Tfap2c expression on D16 (Fig. S3). Overall, expression of Tfap2c by the placental Jz was ~20% lower in females compared to males.

In addition to maternal re-activation of Igf2 and downregulation of H19, Jz-ΔICR1 will also result in expression of the normally-silenced maternal mir-483 (located within intron 2 of Igf2), and reduced expression of mir-675 (located within exon 1 of H19). Although no miR-483 targets have been identified, some for miR-675 have been reported, including Igf1r, Rapgap1, Egr3 and Slc44a1 (Keniry et al., 2012). However, the expression of these by the placental Jz was not significantly affected by Jz-ΔICR1 (Fig. S2A and Fig. S4). Although Rapgap1 was expressed at a lower level in females compared to males, an effect significant by pairwise comparison for control fetuses (Fig. S4).
Emerging studies demonstrate a role for imprinted genes in regulating placental endocrine capacity (John, 2013; 2017), with constitutive gene manipulations showing that the maternally expressed genes Ascl2 and Phlda2 restrict Jz size (Tunster et al., 2015, 2016), and paternally expressed genes Peg3 and Igf2 appearing to enhance Jz size (Esquiliano et al., 2009; Tunster et al., 2018). Furthermore, we recently reported that conditional loss of Igf2 in cells of the Jz affects placental endocrine capacity in a sexually dimorphic manner (Aykroyd et al., 2020). In the present study, we generated a novel loss of imprinting (LOI) model to investigate the role of imprinting of the ICR1 domain in modulating placental endocrine capacity. We utilised Tpbpa-Cre-mediated deletion of ICR1 to drive LOI of the ICR1 domain specifically in cells of the Jz of the mouse placenta (Jz-ΔICR1). Quantification of gene expression in isolated Jz samples revealed a ~30% increase in Igf2 expression and a concomitant ~40% reduction in H19 expression within the Jz following maternal transmission of the floxed allele. Although the magnitude of changes in Igf2 and H19 expression may partly reflect the presence of Tpbpa-negative cells in isolated Jz samples, a similar level of Igf2 reactivation and H19 suppression has been seen in another study involving a 1.6 kb deletion spanning ICR1 (Thorvaldsen et al., 1998). Moreover, our findings reinforce the idea that there may be additional mechanisms controlling the placental expression of these imprinted genes from the maternal allele (Kaffer et al., 2001; Nordin et al., 2014; Sasaki et al., 2000). Importantly, correct spatial localisation of Igf2 and H19 transcripts was maintained in Jz-ΔICR1 placentas, and there was no effect on expression of Igf2 or H19 in the Lz.

Whilst placental weight was unaffected, Jz-ΔICR1 resulted in a slight reduction in Lz and Db volumes with a concomitant increase in Jz volume in placentas of both male and female fetuses. When analysed independent of sex, the increased Jz volume was attributable to increased volumes of all three Jz constituent cell types (SpT, GC and TGC), with no effect on average size of SpT or GC. This is consistent with findings in the H19Δ13 model, in which cell size was unaltered (Esquiliano et al., 2009), and suggests that the increased volume of Jz cell types is related to an expansion in cell number. Whilst IGF2 may have an anti-apoptotic function (Sferruzzi-Perri et al., 2017), the level of the apoptotic marker caspase-3...
was unaltered in the Jz by Jz-ΔICR1. However, there was more than a 3-fold increase in the percentage of Jz cells positive for the proliferation marker Ki67 in Jz-ΔICR1 placentas compared with controls. In addition to the well-established role of IGF2 in promoting trophoblast proliferation (Chen et al., 2016; Forbes et al., 2008), H19 has also been linked to regulating placental cell proliferation. A Jz specific manipulation that results in upregulated H19 expression also led to a 75% reduction in the number of Ki67 positive cells compared to control placentas at day 12.5 (Sharma et al., 2016). Taken together, these data show that the enhancement of Jz growth observed with Jz-ΔICR1 is due to an increase in proliferation and not a change in Jz cell size or apoptosis, which occurs as result of both Igf2 and H19 mis-expression.

In addition to mediating the transfer of nutrients from mother to fetus, the placenta also stores glucose as glycogen (Lefebvre, 2012; Tunster et al., 2020). These placental glycogen stores are thought to provide an important source of energy to support fetal growth during late gestation (Coan et al., 2006). Whilst Jz-ΔICR1 did not affect expression of key glycogenesis pathway genes measured, or weight adjusted glycogen concentration within the Jz, total placental glycogen content was increased. These findings are consistent with prior work showing that manipulation of the Igf2-H19 locus affects placental glycogen levels (Esquiliano et al., 2009; Lopez et al., 1996). The effect of Jz-ΔICR1 to increase total placental glycogen was most pronounced for female fetuses, and overall, reflected greater GC number. These data indicate that imprinting of ICR1 in the Jz acts, at least in part, to restrict placental glycogen storage, thus reducing the necessity of the mother to invest resources that she may otherwise require for maintenance of health.

Expression of markers of Jz cell lineages and various endocrine related genes were largely unaltered at the cellular level by Jz-ΔICR1. However, since the abundance of the cell types expressing these genes is increased, total placental expression/output of such endocrine mediators would be predicted to be increased. A notable exception to this was Psg23 expression, which increased >2-fold in the placental Jz of both males and females in response to Jz-ΔICR1. PSGs are thought to have a predominantly immune-modulatory function, and PSG23 is one of the most abundant PSGs expressed in the mouse placenta in late gestation (McLellan et al., 2005). Human PSGs induce the production of anti-inflammatory cytokines in vitro (Snyder et al., 2001). Additionally, murine PSG23 and human PSG1 share a common function in promoting feto-placental blood supply via induction of vascular remodelling and angiogenesis (Lisboa et al., 2011; Wu et al., 2008). Given the up-
regulation of \textit{Psg23} expression, future work should evaluate whether maternal spiral artery remodelling, inflammatory cytokine production, placental vascularisation and utero-placental blood flow may be altered by Jz-ΔICR1. However, the expression of the angiogenesis regulators \textit{Flt1} and \textit{Vegfa} were unaffected by Jz-ΔICR1 in this study. Taken together, Jz-ΔICR1 appears to increase placental endocrine capacity and function through two mechanisms; firstly, by increasing the abundance of the endocrine cell lineages (SpT, GC and TGC), and secondly by driving increased expression of \textit{Psg23} at the cellular level.

The mechanism underlying enhanced \textit{Psg23} with Jz-ΔICR1 is unclear. Interestingly, \textit{Tpbpa}\textsuperscript{-}Cre-mediated ablation of the transcription factor \textit{Tfap2c} resulted in a reduction in Jz size, with increased expression of \textit{H19} and decreased expression of \textit{Psg23} in the Jz (Sharma et al., 2016). However, expression of \textit{Tfap2c} was unaltered in response to Jz-ΔICR1, which argues against a role for altered \textit{Tfap2c} in our model. Maternal re-activation of \textit{Igf2} and downregulation of \textit{H19}, will also result in expression of the normally-silenced maternal \textit{mir-483} (located within intron 2 of \textit{Igf2}), and reduced expression of \textit{mir-675} (located within exon 1 of \textit{H19}). miRNAs can regulate gene expression and have been implicated in Jz cell proliferation and differentiation (Sharma et al., 2019). As miR-675 has been linked with placental growth suppression (Keniry et al., 2012), dysregulation of miR-483 or miR-675 could contribute to the phenotypes observed in response to Jz-ΔICR1. However, we did not observe any alterations in expression of several miR-675 targets, which include \textit{Igrf1}, in response to Jz-ΔICR1. Furthermore, expression of \textit{Psg23} is unaltered in the \textit{H19}\textsuperscript{-} model (Keniry et al., 2012), suggesting that \textit{Psg23} is not a target of miR-675. No miR-483 targets have been identified to date, thus the contribution of perturbed miR-483 expression to the phenotype observed with Jz-ΔICR1 requires study.

We wanted to further explore the underlying molecular mechanisms through which imprinting of ICR1 may exert a regulatory influence on placental endocrine capacity. Since IGF2 is elevated ~30\% in the Jz as a result of Jz-ΔICR1, and the phenotype in the \textit{H19}\textsuperscript{-} model was attributed to elevated IGF2 (Leighton et al., 1995a), we focused our attention on the abundance of receptors that bind to, and signalling pathways downstream of, IGF2. In females, protein levels of INSR, PI3K-P85, phosphorylated AKT and total AKT were increased alongside decreased levels of total GSK3 in the placental Jz response to Jz-ΔICR1. As the PI3K-AKT signalling pathway inhibits GSK3, which is a negative regulator of glycogen synthesis (Cross et al., 1995; Diehl et al., 1998), our findings are consistent with increased placental glycogen synthesis in Jz-ΔICR1 females. Deletion of a negative
regulator of PI3K-AKT signalling has been shown to increase placental Jz size in mice (Church et al., 2012). This signalling pathway is also implicated in the proliferation and differentiation of individual trophoblast cells in the placenta, notably GC and TGC (Kent et al., 2010; Lee et al., 2019; Sferruzzi-Perri et al., 2016). Thus, enhanced PI3K-AKT activation (via INSR) could also explain the increase in Jz formation in females with Jz-ΔICR1.

In males with Jz-ΔICR1, there was no increase in the abundance of signalling receptors for IGF2 (there was even a trend for reduced IGF1R), and abundance of total AKT was decreased and levels of total GSK3 increased in the placental Jz. However, there was a trend for elevated PI3K-P110β, and the ratio of active phosphorylated to total AKT was increased, whilst the ratio of inactive phosphorylated to total GSK3 increased in Jz-ΔICR1 males. These changes all suggest enhanced PI3K-AKT signalling. However, unlike in females, this enhanced PI3K-AKT signalling was not associated with an increase in total placental glycogen or GC and TGC abundance in Jz-ΔICR1 males. These data collectively suggest that the precise mechanism through which activation of PI3K-AKT signalling occurs, or the existence of other signalling pathways, are important for modulating glycogen levels and Jz morphogenesis in the female and male placenta. Indeed, males, but not females, showed an increased level of phosphorylated MAPK, phosphorylated P38 and phosphorylated to total P38 ratio in response to Jz-ΔICR1. Members of the MAPK pathway are activated by IGF2 to promote cell proliferation (Forbes and Westwood, 2008). P38 signalling also regulates programmed cell death pathways (Cheng and Feldman, 1998; Li et al., 2003) and is important for murine placental Jz formation, particularly SpT differentiation (Mudgett et al., 2000). While there was no change in apoptosis with Jz-ΔICR1, elevated abundance and activation of MAPK pathway could also explain the enhanced in Jz formation in males in response to Jz-ΔICR1. Changes in Jz protein abundance with Jz-ΔICR1 were not linked to alterations in gene expression, highlighting a role for post-transcriptional regulation in mediating the changes seen for males and female fetuses, which require study in further work.

Previous studies manipulating the expression of genes within the ICR1 domain have demonstrated profound effects on fetal growth. For instance, maternal inheritance of the H19Δ13 allele, which deletes both H19 and ICR1 and results in maternal reactivation of Igf2, causes a 27% increase in fetal weight (Leighton et al., 1995a). Similarly, maternal inheritance of a deletion spanning only the ICR1 results in a 17% increase in neonatal weight (Thorvaldsen et al., 1998), whereas targeted deletion of H19 (H19Δ3), which results in
partial (~25%) reactivation of maternal \( \text{Igf2} \), enhanced fetal weight by ~8% (Ripoche et al., 1997). Despite a ~30% increase in \( \text{Igf2} \) expression in the Jz and the enhanced placenta endocrine and glycogen storage capacity, fetal weight was not increased in our Jz-\( \Delta \text{ICR1} \) model. There are several potential explanations for this observation. Firstly, deletion of ICR1 was specifically targeted to cells of the Jz, and \( \text{Igf2} \) expression in the Lz or fetus (which are unaltered) may be relatively more important for fetal growth. This notion is in line with previous studies using manipulations that only affect one compartment of the placenta or fetus (Aykroyd et al., 2020; Sandovici et al., 2019; Sferruzzi-Perri et al., 2011). Secondly, we assessed impacts on fetal weight on day 16 of pregnancy only. This day was chosen as it is when the placental Jz is at its largest size (Coan et al., 2006) and mouse dams are most insulin resistant to favour fetal nutrient supply (Musial et al., 2016). Hence, work is required to explore if changes in placental endocrine phenotype with Jz-\( \Delta \text{ICR1} \) offer benefit when the fetus enters its exponential growth phase in the lead up to term. Thirdly, we generated litters of mixed genotypes, such that both control and Jz-\( \Delta \text{ICR1} \) conceptuses were exposed to the same \textit{in utero} environment. Whilst this approach serves to normalise variations in the maternal environment, control littersmates will be exposed to the potentially altered \textit{in utero} environment caused by enhanced endocrine capacity of Jz-\( \Delta \text{ICR1} \) placetas and may not show the normal pattern of fetal growth. Indeed, the phenotype of genetically wild-type littersmates has been shown to be influenced by mutant littersmates with phenotypes affecting placental endocrine function (López-Tello et al. 2019; Tunster et al. 2016). Future work may address any possible dilution of an effect on fetal growth by undertaking comparisons between litters comprised entirely of Jz-\( \Delta \text{ICR1} \) or wild-type conceptuses.

In light of accumulating evidence of sexual dimorphism in placental adaptations to genetic and/or environmental perturbations (Aykroyd et al., 2020; Barke et al., 2019; Kalisch-Smith et al., 2017; Napso et al., 2019; Rosenfeld, 2015) and offspring outcomes (Christoforou and Sferruzzi-Perri, 2020; Dearden et al., 2018; Rodgers and Sferruzzi-Perri, 2021), we accounted for fetal sex in our analyses. Whilst Jz size was increased in placetas of both male and female conceptuses in response to Jz-\( \Delta \text{ICR1} \), the underlying mechanisms appear to exhibit a degree of sexual dimorphism. For instance, the increase in Jz volume of Jz-\( \Delta \text{ICR1} \) females was attributed to increased volumes of all three Jz cell types, whereas statistical significance was achieved only for SpT in Jz-\( \Delta \text{ICR1} \) males. Furthermore, abundance of individual components of the PI3K-AKT and MAPK signalling pathways in the Jz with Jz-\( \Delta \text{ICR1} \) was dependent on fetal sex. We also found that placental weight and Jz size were greater in males compared to females, alongside increased expression of several Jz lineage markers (\( \text{Gjb3}, \text{Pcdh12}, \text{Hand1} \)), glycogen synthesis genes (\( \text{Gys1}, \text{Gbe1}, \text{Gsk3} \)),...
growth regulators (Insr, N-ras, Mek1, Tfap2c, Rapgap1) and endocrine related genes (Stard1, Prl2c2, Prl3d1, Psg23), irrespective of genotype. Our findings further emphasise the need to control or account for fetal sex in studies of fetal and placental developmental physiology.

In summary, Jz-ΔICR1 enhances endocrine cell formation and Jz hormone expression. Whilst this phenotype is observed in both sexes, the signalling pathway response mechanisms attributable to these alterations are sexually-dimorphic. Moreover, the expansion of the Jz occurs at the expense of Lz size which is although not associated with a significant alteration in placental or fetal weight. The influence of altered placental phenotype with Jz-ΔICR1 on fetal and maternal physiology is yet to be determined. Studies of the human placenta have reported perturbations in the regulation of the Igf2-H19 locus and alterations in the expression/abundance of IGF2 signalling factors and placental hormones in pregnancy complications including gestational diabetes, fetal growth restriction and large for gestational age (Laviola et al., 2005; Le et al., 2013; Liao et al., 2017; Ngala et al., 2017; Su et al., 2016). Therefore, this study may provide valuable insight for understanding the pathogenesis of human pregnancy conditions. In addition, the magnitude of changes in Igf2 and H19 expression seen in this study supports the notion that there may be other regulatory mechanisms controlling the expression of these genes from the maternal allele in the placenta. Finally, our study highlights that maternal and paternal imprinted genes may govern the allocation of resources to the fetus additionally via modulation of placental endocrine function in pregnancy.
**Materials and methods**

**Maintenance of transgenic mice**

The experiments for this study were approved by the University of Cambridge Animal Welfare and Ethical Review Body performed under the UK Home Office Animals (Scientific Procedures) Act 1986. Homozygous TpbpaCre males (Simmons et al., 2007) were mated to heterozygous ICR floxed females (LoxP sites surrounding the ICR; termed ICR1Flox; Srivastava et al., 2000) to generate litters containing fetuses with control and Jz-ΔICR1 placentae (Fig. 1). The transgenic mice were maintained on a C57BL/6NCrl (Charles River, UK) background for >10 generations. Mice were housed under a 12:12h light/dark photocycle, 22°C air temperature and 21% oxygen saturation with access to water *ad libitum* and standard laboratory chow (Rat and Mouse No.3; Special Dietary Services, UK).

**Tissue collection**

Dams were killed by cervical dislocation on day 16 of pregnancy (presence of a copulatory plug denoted day 1 and term occurs ~ day 20). Placentae and fetuses were dissected from the dam uterus and weighed. Placentae were bisected on the short axis, one half was separated into individual Jz and Lz, as described previously by Sferruzzi-Perri et al., 2009, snap-frozen in liquid nitrogen and stored at -80°C for either gene expression or western blotting analysis. The remaining placental half was kept whole and either snap frozen in liquid nitrogen and stored at -80°C for placental glycogen content analysis, or fixed in 4% paraformaldehyde, dehydrated, embedded into paraffin wax and sectioned exhaustively from the mid-line at 8 μm for histological analysis. Fetal tails were collected for PCR to establish the Flox genotype (FPrimer: 5’- CAGGCCTGTCTCCTACCTGAAC-3’, RPrimer: 5’-GCCAGCTTGCTTGGAACCCCTCTT-3’) and sex with Sly genotyping (FPrimer: 5’-GTGGTTCTGTCCACTGC-3’, RPrimer: 5’-GGCCATGTCAAGCGCCCCAT-3’ with a PCR autosomal gene control FPrimer: 5’-TGGTTGCACTTTATCCCTAGAAC-3’, RPrimer: 5’-GCAACATGGCAACTGGAAACA-3’). Placentae with weights closest to the mean value for each litter were selected from each genotype group for analysis.
Stereological analysis

Every 20th paraffin-embedded placental section was stained with haematoxylin and eosin (n = 8 per genotype/sex, across 8 litters). Images of each placental section were captured at 40x magnification using a NanoZoomer 2.0-RS (Hamamatsu, JP). The gross structure of each placental zone (Db, Jz and Lz) and the proportion of cells in the Jz and Db were analysed using the newCAST System (Visiopharm, DK) as described by Aykroyd et al., 2020. The average size of Jz GC was estimated by using the freehand annotation tool in the NDP.view2 viewing software (Hamamatsu, JP) and measuring the area of 100 individual Jz GC in a mid-line placental section (n = 4 per genotype/sex, across 4 litters). The average number of SpT and GC in the placental Jz was determined by dividing the SpT and GC volume by average cell size. A qualitative assessment of Jz interdigitation into the Lz, and Jz boundary integrity with the Db and Lz was also performed (n = 4 per genotype/sex, across 4 litters).

In situ hybridisation

The expression of Igf2, H19, Tpbpa, Prl8a8 and Psg23 were localised in placental sections using in situ hybridisation. Previous studies have described the generation of probes for Igf2 and Prl8a8 (Aykroyd et al., 2020) and Tpbpa (Lescisin et al., 1988). A 659bp region of Psg23 was amplified by PCR from wild-type placental cDNA (primer sequences: 5'-GCTGTGACCCTCTTGACTCT-3', 5'-AAATGCCTCTGCCTGCTAT-3'), cloned into the pDrive vector system (Qiagen, DE), with linearised vector used as template for probe transcription. For H19, the template for probe transcription was generated by PCR amplification of a 405 bp fragment from wild type placental cDNA using primers incorporating a T3 (forward primer: 5'-AATTAACCCTCACTAAAGGGTTGTCGAGAAGCCGTCTGT-3') or T7 (reverse primer: 5'-TAATACGACTCACTATAGGAGGGAGAGAGATGATGAAGT-3') RNA Polymerase binding site, as described by Langford et al., 2018. The amplicon was purified using the Monarch PCR and DNA Cleanup Kit (NEB), and 1 ug used as template for transcription of digoxigenin labelled riboprobes using the DIG RNA Labelling mix (Sigma).

Probe hybridisation was performed overnight at 60°C, as described by Rakoczy et al., 2017. Staining was developed using BM-Purple alkaline phosphatase substrate (Sigma) and sections were counterstained using Nuclear Fast Red (Sigma-Aldrich, US). DIG-labelled
sense riboprobes with identical sequences to the target mRNA were used as negative controls.

**Immunohistochemistry**

Apoptosis and proliferation levels were measured by immunostaining in dewaxed and rehydrated midline placental sections with cleaved caspase-3 (Asp175) (Cell Signalling, US, 9661; 1:200) and Ki67 (Abcam, UK, ab264429; 1:500). Sections were incubated with goat-anti-rabbit secondary antibody (Abcam, UK, ab6720; 1:1000), streptavidin-horseradish peroxidase (Rockland, US, S000-03, 1:250) and stained with 3,3'-Diaminobenzidine (Abcam, UK). Haematoxylin was used as a counterstain before dehydrating and mounting the sections. Caspase and Ki67 positive cells were identified and counted in the placental Jz using NDP.view2 viewing software (Hamamatsu, JP), (n = 3-5 per genotype/sex, across 5 litters). Negative controls were prepared by the omission of primary antibodies.

**Glycogen assay**

Amyloglucosidase was used to indirectly measure glycogen content in bisected placental halves (n = 8 per genotype/sex, across 8 litters), as previously described (Sferruzzi-Perri et al., 2013b).

**Placental gene expression**

Total RNA was extracted and 5 μg reverse transcribed from paired isolated Jz and Lz placental tissues (n = 8-10 per genotype/sex, across 11 litters) using the RNeasy Plus Mini Kit (Qiagen, DE) and the High-Capacity cDNA Reverse Transcription Kit minus RT inhibitor (Applied Biosystems, US), according to manufacturer's instructions. Primer sequences were sourced from publications and reported previously (Aykroyd et al., 2020) or designed using NCBI Primer Blast and produced by Sigma-Aldrich (Table S1). Only primers which produced a PCR product of the desired size, correct sequence and with amplification efficiencies of >85% were used. Samples were measured in duplicate on a 7500 fast real-time PCR machine (Applied Biosystems, US) with MESA Blue SYBR (Eurogentec, BE) under the following conditions: 3 minutes at 95°C then 40 cycles of: 30s at 95°C, 30s at 57°C, 90s at 72°C. The cycle threshold expression values for each gene were normalised to the geometric mean of housekeeping genes *Hprt* and *Ywhaz* for Jz samples and *Hprt* and *Polr2a* for Lz samples. All reference genes were unaltered by genotype or sex. Fold change
was calculated according to the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and represents an estimation of fold change at the cellular level, since expression is assessed in the Jz or Lz in isolation.

**Placental Jz protein expression**

Protein was extracted from approximately 50mg of placental Jz tissue (n = 7 per genotype/sex, across 9 litters) using RIPA buffer (Thermo Scientific, US) containing complete Mini EDTA-free protease inhibitor cocktail mix (Roche, CH). The protein concentration of Jz lysates was determined using the Bicinchoninic Acid protein assay (Thermo Scientific, US). Lysates were diluted to 2.5µg/µl in lysis buffer and 1xSDS, resolved using SDS-PAGE and transferred onto 0.2 µm nitrocellulose membranes (Bio-Rad Laboratories, US). Even protein loading and successful protein transfer was confirmed using Ponceau S stain (Sigma Aldrich) prior to probing with primary antibodies (Table S2). Anti-rabbit secondary antibody tagged to horseradish peroxidase (NA934 Cytiva, US, 1:10000 in 1xTBST with 2.5% milk/BSA) was used for all membranes. Bands were visualised using Scientific SuperSignal West Femto enhanced chemiluminescence (ECL) substrate (Thermo Scientific, US) and imaged using an iBright 1500 Imaging System (Invitrogen, US). Abundance of proteins were quantified using ImageJ analysis software (National Institutes of Health, US) to measure the pixel intensity of protein bands. Protein loading was controlled for by normalising against Ponceau S staining.

**Statistics**

Prior to statistical analysis, a Prisms Grubbs’ test (GraphPad Software Inc.) was performed on all data sets to identify any outliers as a quality check. In the majority of cases, entire datasets did not contain outliers, and if they did, at most a single sample from a group was excluded. Final sample numbers are detailed within each table or figure legend. With the exception of protein abundance analyses, all data were analysed by two-way ANOVA (genotype and sex). If an overall significant effect of sex or genotype was identified, then planned comparisons using two-tailed T-tests were performed. Protein abundance was assessed for males and females separately to maintain a high sample size (n) per group and effect of genotype determined using two-tailed T-tests. Prism (GraphPad Software Inc., US) was used to perform statistical analyses with a significance value of p<0.05. Results are shown as mean +/- SEM, n = number of fetuses or placentae in each group.
Acknowledgements

We are grateful to Miguel Constância and Ionel Sandovici for providing the TpbpaCre and ICR1Flox animals used in this study and staff of the Combined Animal Facility for assistance in animal husbandry.

Competing interests

No competing interests declared.

Funding

This work was supported by a Royal Society Dorothy Hodgkin Research Fellowship, Academy of Medical of Sciences Springboard Grant and Medical Research Council New Investigator grant to ANSP (grant numbers DH130036 / RG74249, SBF002/1028 / RG88501 and MR/R022690/1 / RG93186, respectively). BRLA received stipendiary support from the Cambridge Trust and Wolfson College. SJT was funded by a Next Generation Fellowship from the Centre for Trophoblast Research and an Early Career Grant from the Society for Endocrinology.
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Figure legends

Fig. 1. Jz-ΔICR1 mouse genetic manipulation model. (A) Schematic diagram of the Igf2/H19 gene locus. Imprinting control region (ICR), CCCTC binding factor (CTCF). (B) Breeding strategy to produce litters with control and Jz-ΔICR1 conceptuses. Cre-recombinase (Cre), chromosome (Chr), homozygous (hom), heterozygous (het), triangles represent lox-P sites and arrows represent transcriptional direction.

Fig. 2. Igf2 expression is increased and H19 expression is decreased in the Jz of mouse placentas with Jz-ΔICR1. (A) Expression of Igf2 and H19 in isolated Jz and Lz samples on D16 relative to the geometric mean of housekeeping genes (Hprt and Ywhaz for Jz, Hprt and Polr2a for Lz) using qPCR (n = 9-10 per genotype/sex in Jz and Lz, across 11 litters). Values presented as mean ± SEM with significance assessed by two-way ANOVA and pairwise T-test (p genotype < 0.05 = *, < 0.01 = **). In situ hybridization of (B) Igf2 and (C) H19 in males and females. Black boxes represent the area magnified in the image below. Black bar represents 1 mm. Red bar represents 100 μm.
Fig. 3. Jz-ΔICR1 increases the formation of endocrine cells in the Jz of the mouse placenta. (A) Fetal weight and (B) placental weight of D16 males (Control n = 29 and Jz-ΔICR1 n = 27) and females (Control n = 17 and Jz-ΔICR1 n = 27) from individual pups, across 13 litters. Volume of (C) placental regions and (D) Jz cell types (n = 8 per genotype/sex, across 7 litters). Jz SpT cell (E) size distribution, (F) average size and (G) average number, and Jz GC cell (H) size distribution, (I) average size and (J) average number (n = 4 per genotype/sex, across 4 litters). (K) Volume of Db cell types (n = 8 per genotype/sex, across 7 litters). Values presented as mean + SEM with significance assessed by two-way ANOVA and pairwise T-test (pgenotype < 0.05 = *, < 0.01 = **, < 0.001 = ***, psex < 0.05 = +). Decidua (Db), Junctional zone (Jz), Labyrinth zone (Lz), spongiotrophoblast (SpT), glycogen cell (GC), trophoblast giant cell (TGC).

Fig. 4. Jz-ΔICR1 increases proliferation Jz cell types but does not alter apoptosis levels in the Jz of the mouse placenta. Immunostaining for (A) Ki67 in male and female placentas with (B) the percentage of Ki67 positive cells in the Jz, and immunostaining for (C) cleaved caspase-3 in male and female placentas with (D) the number of cleaved caspase-3 positive cells in the Jz. Data were obtained on D16. The black scale bar represents 25 μm. Values presented as mean + SEM with n = 3-5 per genotype/sex, across 5 litters. Significance assessed by two-way ANOVA and pairwise T-test (pgenotype < 0.05 = *, < 0.01 = **). Spongiotrophoblast (SpT), glycogen cell (GC), trophoblast giant cell (TGC).

Fig. 5. Jz-ΔICR1 increases total placental glycogen content in females, but does not alter placental glycogen concentration or the expression of glycogen synthesis pathway genes in mice. Jz expression of (A) endocrine cell markers and (B) glycogen synthesis pathway genes in Jz samples relative to the geometric mean of housekeeping genes (Hprt and Ywhaz) using qPCR (n = 8-10 per genotype/sex, across 11 litters). (C) Placental glycogen concentration (mg/g) and (D) total placental glycogen content (mg) in males and females with Jz-ΔICR1 (n = 8 per genotype/sex, across 8 litters). Data were obtained on D16. Values presented as mean + SEM with significance assessed by two-way ANOVA and pairwise T-test (psex < 0.05 = +, < 0.01 = ++). (E) PAS stain of glycogen containing cells and in situ hybridization of (F) Tpbpa and (G) Prl8a8 in males and females. Black boxes represent the area magnified in the image below. Black bar represents 1 mm. Red bar represents 100 μm.
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Fig. 7. Jz-ΔICR1 alters the protein expression of IGF2 signalling factors and downstream members of the PI3K-AKT and MAPK pathway in the Jz. (A & B) Representative ponceau shown to indicate protein loading with the abundance, and (C & D) quantification of IGF2 signalling proteins in (A & C) males and (B & D) females. Data were obtained on D16 from n = 7 per sex genotype/sex, across 8 litters. Values presented as mean + SEM with significance assessed by T-test (p < 0.05 = *, p < 0.01 = **, p < 0.01 = ***).

Fig. S1. Negative controls for in situ hybridization and immunohistochemistry experiments in D16 mouse placenta. Sense in situ hybridization probes for (A) H19 and (B) Psg23. Secondary antibody only control used in immunohistochemistry experiments for (C) cleaved caspase-3 and (D) Ki67. Black boxes represent the area magnified in the image below. Black bar represents 1 mm. Red bar represents 100 μm.

Fig. S2. Jz-ΔICR1 does not alter the gene expression of IGF2 signalling factors or downstream members of the PI3K-AKT and MAPK pathways. Expression of (A) IGF2 signalling factors, (B) PI3K-AKT pathway genes and (C) RAS-MAPK-ERK pathway genes in Jz samples relative to the geometric mean of housekeeping genes (Hprt and Ywhaz) using qPCR (n = 8-10 per genotype/sex, across 11 litters). Data were obtained on D16. Values presented as mean + SEM with significance assessed by two-way ANOVA and pairwise T-test (p<0.05 = +).
Fig. S3. Jz-ΔICR1 does not alter the gene expression of Tfap2c. (A) Expression of Tfap2c in Jz samples relative to the geometric mean of housekeeping genes (Hprt and Ywhaz) using qPCR (n = 9-10 per genotype/sex, across 11 litters). Data were obtained on D16. Values presented as mean + SEM with significance assessed by two-way ANOVA and pairwise T-test (psex < 0.05 = +).

Fig. S4. Jz-ΔICR1 does not alter the gene expression of miR-675 targets. (A) Expression of miR-675 target genes in Jz samples relative to the geometric mean of housekeeping genes (Hprt and Ywhaz) using qPCR (n = 8-10 per genotype/sex, across 11 litters). Data were obtained on D16. Values presented as mean + SEM with significance assessed by two-way ANOVA and pairwise T-test (psex < 0.05 = +).
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