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Fetal growth restriction in a genetic model of sporadic Beckwith-Wiedemann Syndrome

Tunster, S.J.[1,2,*], Van de Pette, M.[1,3], Creeth, H.D.J.[1], Lefebvre, L[4] and John, R.M.[1]

[1] Cardiff School of Biosciences, Cardiff University, Cardiff, Wales, CF10 3AX, UK.

[2] Current address: Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, CB2 3EG, UK

[3] Current address: MRC London Institute of Medical Sciences, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, UK

[4] Department of Medical Genetics, Life Sciences Institute, University of British Columbia, Vancouver, BC V6T 1Z3, Canada

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*Corresponding author email: sjt95@cam.ac.uk

Summary statement

A novel genetic mouse model of sporadic Beckwith-Wiedemann syndrome recapitulates placentomegaly but late gestation fetal growth restriction contrasts with fetal overgrowth characteristic of BWS.

Abstract

Beckwith-Wiedemann syndrome (BWS) is a complex imprinting disorder involving fetal overgrowth and placentomegaly and associated with a variety of genetic and epigenetic mutations affecting expression of imprinted genes on human chromosome 11p15.5. Most BWS cases are linked to loss of methylation at the Imprint Control Region 2 (ICR2) within this domain, which in mice regulates the silencing of several maternally expressed imprinted genes. Modelling this disorder in mice is confounded by the unique embryonic requirement for *Ascl2* which is imprinted in mice but not humans. To overcome this issue, we generated a novel model combining a truncation of distal chromosome 7 allele (DelTel7) with transgenic rescue of *Ascl2* expression. This novel model recapitulated placentomegaly associated with BWS but did not lead to fetal overgrowth.

Introduction

Beckwith-Wiedemann syndrome (BWS; MIM #130650) is a complex imprinting disorder associated with a range of growth and developmental phenotypes, including overgrowth, macroglossia, abdominal wall defects and an increased frequency of childhood tumours (Brioude et al., 2018; Lapunzina, 2005; Weksberg et al., 2010) Historically, BWS has been diagnosed in the presence of three or more “major” criteria, including: abdominal wall defects, macroglossia (enlarged tongue), macrosomia (birth weight >97th percentile), ear creases/pits and visceromegaly (enlarged abdominal organs). BWS may also be diagnosed on presentation with two major criteria and at least one “minor” criterion, including placental defects, placentomegaly, neonatal hypoglycaemia and cardiomegaly (Ounap, 2016; Weksberg et al., 2010). However, a recent Consensus Statement proposes a re-evaluation of diagnostic criteria, describing several cardinal features (including macroglossia, lateralised overgrowth and placental mesenchymal dysplasia) alongside additional suggestive features (including placentomegaly and fetal overgrowth) (Brioude et al., 2018). The prevalence of BWS is estimated at 1 in 13,700 live births (Thorburn et al., 1970). The majority of cases occur sporadically, while approximately 15% are inherited, for instance via loss of a functional maternal *CDKN1C* allele (Weksberg et al., 2010; Weksberg et al., 2005; Weksberg et al., 2003).

BWS is caused by genetic or epigenetic mutations that disrupt expression of one or more imprinted genes, which, unlike most autosomal genes, are expressed predominantly from one parental allele (Ferguson-Smith and Surani, 2001). The parent-of-origin-specific expression of imprinted genes is regulated through mechanisms that include differential methylation and expression of long non-coding RNAs (Delaval and Feil, 2004; Koerner et al., 2009; O'Neill, 2005). Over 100 imprinted genes have been identified in mice, with around half of these known to be also imprinted in humans (Ishida and Moore, 2013). Most imprinted genes have critical roles in regulating fetal and/or placental growth (Cleaton et al., 2014; Tunster et al., 2013), with the parental conflict hypothesis predicting that paternally expressed imprinted genes promote growth and maternally expressed imprinted genes restrict growth (Moore and Haig, 1991).

BWS results from genetic or epigenetic defects within a ~1 Mb imprinted region of human chromosome 11p15.5 (Koufos et al., 1989; Ping et al., 1989). Imprinting of genes within this domain is associated with differential methylation of two imprinting control regions; IC1 and IC2 (also known as ICR1 and ICR2) (Du et al., 2003; Du et

al., 2004). Methylation of the paternal ICR1 suppresses the non-coding *H19* RNA whilst permitting expression of the growth-enhancing *IGF2*. On the maternal chromosome, absence of ICR1 DNA methylation is associated with expression of *H19* and suppression of *IGF2* (reviewed in Ideraabdullah et al., 2008). In contrast, maternal methylation of ICR2 prevents transcription of the long non-coding RNA *KCNQ1OT1*, which is predicted to permit the expression of several genes, including the growth-limiting *CDKN1C* and *PHLDA2*. On the paternal chromosome, absence of ICR2 methylation permits transcription of *KCNQ1OT1* and is associated with silencing in *cis* of the imprinted genes in the region (Du et al., 2004). Several genetic and epigenetic defects have been associated with BWS, including: paternal uniparental disomy (pUPD), gain of ICR1 methylation, loss of ICR2 methylation and mutations within the *CDKN1C* coding region (Weksberg et al., 2010; Weksberg et al., 2005; Weksberg et al., 2003). Whilst maternally inherited *CDKN1C* mutations account for nearly half of familial cases, only ~5% of sporadic cases are associated with such mutations (Lam et al., 1999; Lee et al., 1997). Instead, the majority of sporadic cases are attributable to loss of ICR2 methylation, which effectively silences expression of several maternally expressed genes regulated by *KCNQ1OT1*, including *CDKN1C* and *PHLDA2* (Gaston et al., 2001; Lee et al., 1999; Smilinich et al., 1999).

Human 11p15.5 is syntenic with mouse distal chromosome 7, with the exception that in humans ICR1 is located towards the telomere and ICR2 towards the centromere, whereas in the mouse this orientation is reversed. Despite conservation of the BWS region between species, modelling BWS in mice remains a complex undertaking. Loss of function of *CDKN1C*, seen most often in familial BWS, has been modelled by the generation of three independent *Cdkn1c* mutant alleles which recapitulate some aspects of the disorder (Takahashi et al., 2000; Yan et al., 1997; Zhang et al., 1997). However, these models are of limited relevance as BWS is typically sporadic and predominantly associated with loss of methylation at ICR2, which is predicted to lead to the silencing of all the maternally expressed genes within the domain.

The generation of a mouse model possessing a truncation of distal chromosome 7 (DelTel7) provided an important first step towards creating a mechanistic model of loss of expression of imprinted genes associated with loss of ICR2 methylation in human (Oh et al., 2008). The DelTel7 truncation encompasses the entire ICR2 imprinted domain and an additional ~20 non-imprinted genes located at the telomeric end of mouse distal chromosome 7, but with imprinted expression of

the ICR1 maintained (Fig. 1). Paternal inheritance of the ~2.6 Mb DelTel7 deletion allele caused no adverse phenotype, consistent with the paternally silenced status of protein coding genes within the domain as well as absence of haploinsufficiency effects. However, maternal inheritance of the deletion resulted in embryonic lethality by E10.5 (Oh et al., 2008). Placentae of maternal DelTel7 heterozygotes were characterised by a loss of junctional zone and expanded giant cell layer, a phenotype reminiscent of *Ascl2* loss of function (Guillemot et al., 1994; Oh et al., 2008). Indeed, reactivation of the paternal IC2 domain by paternal transmission of an IC2 KO allele (KvDMR1 KO) was sufficient to rescue embryonic lethality and restore placental structure, thus attributing the lethality and placental defects to loss of expression of one or more genes within the IC2 domain (Oh-McGinnis et al., 2010).

A key difference in imprinting between human and mouse in the BWS region is the status of *Ascl2*/*ASCL2*, which in mice is maternally expressed, but escapes imprinting in humans (Miyamoto et al., 2002; Monk et al., 2006). The biallelic expression of *ASCL2* in humans may explain survival of human conceptuses with ICR2 hypomethylation, with *ASCL2* expression maintained from the active paternal allele, whereas loss of IC2 imprinting in the mouse completely ablates *Ascl2* expression. We recently reported the generation of a transgenic mouse carrying a BAC transgene spanning the *Ascl2* gene (*Ascl2*^{BAC}) (Reed et al., 2012; Tunster et al., 2016). Here, we asked whether transgenic *Ascl2* expression could rescue the embryonic lethality in the context of maternal DelTel7 inheritance, to allow a later characterisation of this model.

Results

Restoring *Ascl2* expression rescues DelTel7 lethality

We first explored whether restoring *Ascl2* gene expression could rescue the embryonic lethality associated with deletion of the maternal IC2 domain by mating hemizygous males carrying *Ascl2*^{BAC} with heterozygous females that inherited the DelTel7 allele from their father. Full rescue should result in viability beyond E10.5 of three genotypes in equal proportions: fully wild-type embryos inheriting neither genetic alteration; *Ascl2*^{BAC} embryos inheriting only the transgene from their father; and DelTel7; *Ascl2*^{BAC} double mutants (called DelTel7^{BAC}) inheriting the DelTel7 allele from their mother and *Ascl2*^{BAC} from their father. DelTel7 embryos inheriting only the deletion allele should not be observed beyond ~E10.5 (Oh et al., 2008). Twenty-two litters were analysed at E14.5, comprising a total of 158 viable conceptuses of which

35 were DelTel7^{BAC} (Table 1) and twenty-six litters were analysed at E18.5 comprising 147 viable conceptuses of which 30 were DelTel7^{BAC} (Table 1). The presence of the transgene successfully, although not completely, rescued lethality imposed by loss of function of the maternally expressed genes within the IC2 domain allowing a phenotypic assessment of the rescued conceptuses.

Placentomegaly and fetal growth restriction of DelTel7^{BAC} conceptuses

Two diagnostic characteristics of BWS are fetal overgrowth and placentomegaly at term. Consistent with this, DelTel7^{BAC} placentae (P) were significantly heavier than those of control littermates at both E14.5 (114%; $p = 6.26 \times 10^{-10}$) and E18.5 (130%; $p = 1.16 \times 10^{-14}$) (Fig. 2A). However, whilst there was no difference in fetal (F) weights at E14.5, DelTel7^{BAC} fetuses were significantly lighter than controls at E18.5 (88%; $p = 2.13 \times 10^{-9}$) (Fig. 2B). Consequently, the F:P ratio, an approximation of placental efficiency (Fowden et al., 2009), was significantly reduced at both E14.5 (89%; $p = 0.0167$) and E18.5 (68%; $p = 3.95 \times 10^{-22}$) (Fig. 2C).

Progressive loss of junctional zone in DelTel7^{BAC} placentae

Growth restriction of DelTel7^{BAC} fetuses occurred late in gestation typically indicative of an extrinsic cause, such as placental insufficiency. To further investigate this possibility, we undertook a detailed characterisation of DelTel7^{BAC} placentae. The mature mouse placenta comprises three structurally and functionally distinct layers. The maternal decidua (Dec) forms from uterine cells in response to implantation, the endocrine junctional zone (Jz) is responsible for synthesis and secretion of signalling factors, and the labyrinth zone (Lz) is responsible for nutrient and gas transfer (Watson and Cross, 2005). H&E staining of placental midline sections revealed a breakdown in the boundary of the Jz with both the Lz and Dec at E14.5 and E18.5 (Fig. 3A, B). The Jz is primarily comprised of glycogen trophoblast cells (GlyT) and the endocrine spongiotrophoblast (SpT). Periodic acid Schiff staining, which stains GlyT, demonstrated a mislocalisation and increased migration of glycogen cells to the decidua at E14.5, although no overt difference in staining was observed at E18.5 (Fig. 3C, D). *In situ* hybridisation with a riboprobe for the Jz marker *Tpbpa* further demonstrated the loss of Jz integrity at E14.5 and revealed a substantial loss of Jz staining at E18.5 (Fig. 3E, F). Biochemical quantitation of placental glycogen stores at E14.5 identified a modest 27% increase in total placental glycogen ($p = 2.61 \times 10^{-3}$), but this was normalised at E18.5 (Fig. 4A). When adjusted for placental weight,

DelTel7^{BAC} placentae did not accumulate significantly more glycogen per gram of placenta at either stage (Fig. 4B).

Altered gene expression in DelTel7^{BAC} placentae

The mature mouse placenta comprises at least nine distinct trophoblast sub-types (Gasperowicz et al., 2013; John and Hemberger, 2012), each of which is characterised by a unique gene expression profile and spatial organisation. Expression analysis of cell-type-specific gene markers facilitates an assessment of the relative contribution of the various trophoblast lineages to the placenta. Consistent with the gross histological assessment of placental structure (Fig. 3), expression of the Jz marker *Tpbpa* was reduced to 41% of wild type levels at E18.5, with a similar trend observed at E14.5 (64%), although without achieving statistical significance (p = 0.0557) (Fig. 5A). Expression of *Flt1*, which is predominantly expressed in the Jz, was reduced to 46% and 56% of wild type levels at E14.5 and E18.5 respectively (Fig. 5A). Expression of genes that are specifically (*Prl8a8*) or predominantly (*Psg17*, *Psg18*, *Psg19*, *Psg21*) expressed in the SpT was substantially diminished at both E14.5 and E18.5, with expression ranging between 21% and 45% of wild type levels (Fig. 5A). In contrast, genes expressed either specifically or predominantly in GlyT were either elevated or unaltered in DelTel7^{BAC} placentae. Expression of *Pcdh12*, an early marker of GlyT, was increased 84% at E14.5, with a similar trend at E18.5, whereas *Gjb3*, a marker of mature glycogen cells (Coan et al., 2006), was unaltered at both E14.5 and E18.5. Expression of *Prl7b1*, a marker of migratory GlyT (Simmons et al., 2008b), was elevated 2-fold at E14.5, consistent with the pattern of PAS staining previously described (Fig. 3B). *Prl6a1*, a marker of non-migratory GlyT (Simmons et al., 2008b), exhibited a trend for increased expression at E14.5, although without achieving statistical significance, with unaltered expression at E18.5 (Fig. 5A). The reduced expression of Jz markers in DelTel7^{BAC} placentas is consistent the *Cdkn1c*$^{-/+}$ placenta, in which *Tpbpa*, *Prl8a8* and *Flt1* were all downregulated at E15.5 (Tunster et al., 2011).

The boundary between the Jz and Dec is marked by a discontinuous layer of Parietal Trophoblast Giant Cells (P-TGC). An additional four Trophoblast Giant Cell (TGC) sub-types have been described, with spiral artery TGCs (SpA-TGCs) lining maternal spiral arteries as they enter the implantation site, canal-TGCs (C-TGCs) lining maternal blood canals that traverse the Jz and Lz, and sinusoidal TGCs (S-TGCs) replacing the endothelial lining of maternal blood sinuses in the Lz (Simmons et al., 2007). Finally, the recently described channel TGCs (Ch-TGC) line venous

channels that traverse the Jz carrying blood away from the placenta (Gasperowicz et al., 2013). Expression of *Prl3b1*, which is expressed in SpT in addition to P-, C-, S- and Ch-TGCs, was reduced to 45% of wild type levels at E14.5, but not significantly altered at E18.5 (Fig 5B). *Hand1*, which is expressed in at least four TGC lineages (Simmons et al., 2007) (expression in Ch-TGCs has not been investigated) was unaltered at both E14.5 and E18.5. Expression of *Ctsq*, which is expressed in S-TGCs and Ch-TGCs (Gasperowicz et al., 2013; Simmons et al., 2007), was expressed at 50% of wild type levels at E14.5, although without reaching statistical significance ($p = 0.140$), with expression normalised by E18.5 (Fig. 5B). *Prl2c,* which is expressed in SpT in addition to P-, SpA-, C-, and Ch-TGCs, was expressed at 67% of wild type levels at E14.5 but was unaltered at E18.5 (Fig. 5B). Taken together, these gene expression data are indicative of an early loss of the endocrine SpT population that persists to term, with a transient increase in GlyT at mid-gestation that is normalised by term.

Elevated expression of syncytiotrophoblast markers

Trophoblast cells in the Lz are arranged in a trilaminar structure, with a layer of S-TGCs replacing maternal endothelial cells, adjacent to which is a bilayer of multinucleated syncytiotrophoblast cells (SynT-I and SynT-II) formed by cell fusion. The endothelial lining of fetal vessels remains intact and lies adjacent to SynT-II (Rossant and Cross, 2001; Simmons and Cross, 2005; Simmons et al., 2008a). Expression of *Flk1*, which is expressed in fetal endothelium (Hirashima et al., 2003), was reduced to 71% of normal levels at E14.5, but was unaltered at E18.5. Expression of *Dlx3*, which is widely expressed in all Lz trophoblast (Simmons et al., 2008a), was unaltered at both stages. *Syna* and *Ly6e*, which are expressed predominantly in SynT-I (Hughes et al., 2013; Simmons et al., 2008a), were unaltered at E14.5, with an 80% increase in *Syna* expression at E18.5, whilst *Ly6e* expression remained unaltered at this stage (Fig. 5C). Expression of *Gcm1* and *Synb*, both markers of SynT-II (Simmons et al., 2008a), were unaltered at E14.5, but were elevated ~10-fold and ~3-fold at E18.5 respectively (Fig. 5D). DelTel7^{BAC} placentae were therefore characterised by defects in both fetal-derived placental regions.

Excluding a role for elevated *Ascl2*

In our initial work with *Ascl2*^{BAC} we reported that *Ascl2* was expressed at 2.7-fold endogenous levels in the small intestine (Reed et al., 2012), but more recently we reported that placental expression exceeds by 6-fold the endogenous levels (Tunster et al., 2016). Thus, a potential shortcoming of this model is the concomitant over-expression of *Ascl2* in the context of loss of expression of the IC2 domain genes. However, we have also demonstrated that the placental defects associated with elevated *Ascl2* was dependent upon *Phlda2*, with *Ascl2* unable to restrict the SpT on a *Phlda2* null background (Tunster et al., 2016). We therefore hypothesised that excess *Ascl2* would have no phenotypic consequence in the context of maternal inheritance of the DelTel7 deletion allele, in which expression of *Phlda2* is ablated. To further explore this hypothesis, we investigated the phenotypic outcomes associated with *Ascl2*^{BAC} combined with loss of function of either *Phlda2* or *Cdkn1c* in isolation.

Transgenic *Ascl2*^{BAC} males were mated with females carrying either a *Phlda2* or *Cdkn1c* loss-of-function allele. Twenty-six litters were generated from crossing the *Phlda2* null line with *Ascl2*^{BAC} males, comprising 187 viable conceptuses and twenty litters were generated from crossing the *Cdkn1c* null line with *Ascl2*^{BAC} males, comprising 150 viable conceptuses. All genotypes were recovered at the expected frequency at E18.5 (Table 2).

Consistent with previous reports (Frank et al., 2002; Tunster et al., 2015; Tunster et al., 2011), both *Phlda2*$^{-/+}$ (122%; $p = 6.73 \times 10^{-12}$) and *Cdkn1c*$^{-/+}$ (136%; $p = 1.00 \times 10^{-20}$) placentae were significantly heavier than control littermates. Similarly, both *Phlda2*$^{-/+BAC}$ (134%; $p = 1.00 \times 10^{-16}$) and *Cdkn1c*$^{-/+BAC}$ (130%; $p = 6.66 \times 10^{-16}$) placentae were significantly heavier than those of control littermates (Fig. 6A, B). Whilst *Phlda2*$^{-/+BAC}$ placentae were heavier than *Phlda2*$^{-/+}$ (110%; $p = 1.82 \times 10^{-4}$), there was no difference in placental weight between *Cdkn1c*$^{-/+}$ and *Cdkn1c*$^{-/+BAC}$ (96%; $p = 0.0946$) (Fig 6A, B). Importantly, fetal weights of *Phlda2*$^{-/+}$ (102%; $p = 0.604$) and *Phlda2*$^{-/+BAC}$ (96%; $p = 0.0752$) did not differ significantly from that of control littermates, although *Phlda2*$^{-/+BAC}$ fetuses were slightly lighter than *Phlda2*$^{-/+}$ (94%; $p = 0.00416$) (Fig. 6C). We previously reported that *Cdkn1c*$^{-/+}$ embryos on a 129 genetic background were 15% heavier than control littermates at E15.5 and 8% heavier at E18.5, with this slowdown in fetal growth trajectory late in gestation attributable to the associated placental defects (Tunster et al., 2011). On this 129 x CD1 genetic background, *Cdkn1c*$^{-/+}$ embryos were 14% heavier than control littermates at E18.5 (*p*

= 6.21 x 10^{-6}), with *Cdkn1c*$^{-/+BAC}$ 9% heavier than control (p = 0.0232), although fetal weight did not differ significantly between *Cdkn1c*$^{-/+}$ and *Cdkn1c*$^{-/+BAC}$ (95%; p = 0.155) (Fig 6D). F:P ratios were significantly reduced for all genotypes as a result of substantial placentomegaly (with the exception of *Ascl2*^{BAC}) (Fig. 6E, F).

Similarly, *Ascl2*^{BAC} did not overtly affect placental phenotype in the context of loss of function of *Phlda2* or *Cdkn1c*. For instance, the increased placental glycogen associated with loss of *Phlda2* was not influenced by the presence of *Ascl2*^{BAC} (Fig 6G), and whilst both *Cdkn1c*$^{-/+}$ and *Cdkn1c*$^{-/+BAC}$ placentas exhibited a trend for reduced placental glycogen, this did not achieve statistical significance (Fig 6H). Key lineage markers were typically expressed at normal levels in *Phlda2*$^{-/+BAC}$ placentas, whilst the expression profile of *Cdkn1c*$^{-/+BAC}$ placentas was largely consistent with previous reports of *Cdkn1c*$^{-/+}$ placentas (Tunster et al., 2011). Importantly, consistent with DelTel7^{BAC} placenta, expression of the SynT-I marker *Syna* and the SynT-II marker *Synb* was upregulated in *Cdkn1c*$^{-/+BAC}$ placentas, although upregulation of *Synb* was less severe (2.5 fold vs 10 fold respectively), consistent with the more severe DelTel7^{BAC} phenotype resulting from the combined loss of *Cdkn1c* and *Phlda2*.

Taken together, these data support the conclusion that *Ascl2*^{BAC} does not contribute significantly to FGR in the context of loss of function of *Cdkn1c* or *Phlda2* in isolation, with no adverse effect on *Phlda2*$^{-/+BAC}$ fetal growth and *Cdkn1c*$^{-/+BAC}$ fetuses retaining the overgrowth inferred by loss of function of *Cdkn1c*.

Discussion

This work sought to establish a novel genetic model of sporadic Beckwith-Wiedemann syndrome associated with loss of maternally-expressed genes in the ICR2 imprinted domain. Loss of expression of the IC2 domain imprinted genes was modelled by maternal inheritance of the DelTel7 truncation allele, with co-inheritance of *Ascl2*^{BAC} to rescue the embryonic lethality caused by loss of *Ascl2* in the mouse. Whilst placentomegaly of DelTel7^{BAC} conceptuses was consistent with BWS, we did not recapitulate fetal overgrowth, which was previously considered a defining characteristic of BWS (Brioude et al., 2018; Weksberg et al., 2010). Our findings share some similarity with our previous characterisation of the *Cdkn1c*$^{-/+}$ model of familial BWS. *Cdkn1c*$^{-/+}$ placentae were substantially heavier than controls with a diminished Jz. Whilst fetal overgrowth was apparent at E15.5 and E18.5, this was absent at birth. The Lz of *Cdkn1c*$^{-/+}$ placentae was characterised by large thrombotic lesions, which we reasoned compromised placental function, leading to the loss of fetal overgrowth

during late gestation (Tunster et al., 2011). Similarly, whilst DelTel7^{BAC} placentae were substantially heavier than control placentae, we did not observe fetal overgrowth at either E14.5 or E18.5. Whilst we did not observe the large thrombotic lesions of *Cdkn1c*$^{-/+}$ placentae, the substantially elevated expression of the SynT-II markers *Gcm1* and *Synb* is consistent with a widespread disruption of the trilaminar structure of the Lz. We conclude that the larger placenta associated with both BWS models is unable to support fetal overgrowth as a consequence of severe placental defects that compromise normal placental function. Our data is consistent with loss of function of the maternally expressed genes within the IC2 domain having a more profound consequence for fetal growth potentially due to the tighter epigenetic regulation of these genes in mice relative to the human locus.

We previously reported that early fetal overgrowth in the *Cdkn1c*$^{-/+}$ model of familial BWS was lost towards term (Tunster et al., 2011). We suggested that this could be due to severe placental defects that impair the ability of *Cdkn1c*$^{-/+}$ fetuses to compete for shared maternal resources. Alternatively, given the role of *Phlda2* in regulating placental glycogen stores (Tunster et al., 2010), thought to be important for fetal growth (Coan et al., 2006), we hypothesised that a combined loss of function of the two genes might be required for fetal overgrowth to manifest (Tunster et al., 2011). Here, we were able to distinguish between these two possibilities using the DelTel model combined with our *Ascl2* transgene, a model which more closely recapitulates sporadic BWS associated with loss of ICR2-regulated genes. The absence of fetal overgrowth after combined loss of *Cdkn1c* and *Phlda2* supports our first scenario whereby the severe placental defects as a consequence of loss of *Cdkn1c* prevent late fetal overgrowth. Consistent with this, DelTel7^{BAC} placentae were substantially heavier and possessed a diminished Jz, with significantly elevated expression of the SynT-II markers *Gcm1* and *Synb* at E18.5.

In mice, *Cdkn1c* is fully silenced by layers of epigenetic marks including direct DNA methylation (John and Lefebvre, 2011). However, in humans the locus lacks local DNA methylation on the paternal allele (Diaz-Meyer et al., 2005) and there is “leaky” expression from the paternal *CDKN1C* allele (Diaz-Meyer et al., 2005; Hatada et al., 1996; Matsuoka et al., 1996; Taniguchi et al., 1997). This suggests that late fetal overgrowth observed in BWS might be attributable to this incomplete silencing of the paternal *CDKN1C* allele in humans, which may be sufficient to prevent the marked placental defect.

There are alternative explanations that must be considered, not in the least that the mouse and human placenta differ substantially both structurally and transcriptionally (Carter, 2018; Soncin et al., 2018), which may prevent the accurate modelling of this disorder in mice. The DelTel7 truncation itself may cause fetal growth restriction as it includes haploinsufficiency of approximately 20 biallelically expressed genes located at the telomeric end of distal chromosome 7 (Oh et al., 2008). Arguing against this is the absence of any phenotype associated with paternal inheritance of the DelTel7 allele, in which appropriate expression of the IC1 and IC2 domain genes is maintained, while one copy of the non-imprinted telomeric genes is deleted (Oh et al., 2008). A final possibility is that the combination of the *Ascl2* transgene plus DelTel7 impairs fetal growth. Evidence against this is provided by the data showing no growth restriction when the transgene was combined with loss of function of *Cdkn1c* in isolation.

In summary, we successfully used an *Ascl2* BAC transgene to rescue embryonic lethality associated with maternal inheritance of a truncation allele of distal chromosome 7, thus creating a novel mechanistic model of sporadic BWS. Whilst our model recapitulated the placentomegaly associated with BWS, we did not observe fetal overgrowth. Taken together with all the data in this locus in human and mice, we conclude that it may not be possible to accurately model BWS associated with loss of imprinting at IC2 in mice due either to differences in the epigenetic regulation of the domain between mice and human and/or functional differences between mouse and human placenta.

Materials and Methods

Mice

All animal studies and breeding were approved by the Universities of Cardiff ethical committee and performed under a UK Home Office project license (RMJ). Mice were housed in a conventional unit on a 12-h light–dark cycle with lights coming on at 06.00h with a temperature range of 21°C ± 2 with free access to tap water and standard chow. The DelTel7 strain was generated as described previously (Oh et al., 2008) and was a kind gift from Louis Lefebvre. The *Ascl2*^{BAC}, *Cdkn1c*^{tm1Sje} and *Phlda2*^{loxP} targeted alleles were generated as described previously (Frank et al., 2002; Reed et al., 2012; Zhang et al., 1997). The DelTel7 strain was maintained on the CD1 background by paternal transmission of the deletion allele. The *Cdkn1c* and *Phlda2* null lines were maintained on the 129S2/SvHsd (129) background by paternal

transmission of the targeted allele. The *Ascl2*^{BAC} line was backcrossed by paternal transmission of the transgene to the 129 background for >8 generations prior to mating with females of the DelTel7 strain. The *Ascl2*^{BAC} line was subsequently backcrossed for >6 generations on to the CD1 background prior to mating with females of the *Cdkn1c* and *Phlda2* null lines on the 129S2/SvHsd (129) background.

Weighing studies

Embryonic and placental wet weights were taken at the stated time points after a discernible plug. Embryos and placentae were dissected free from extraembryonic membranes, immersed in ice cold fixative, briefly dried and weighed.

Histological analyses

Placentas were fixed overnight in phosphate-buffered 4% paraformaldehyde (PFA), paraffin-embedded and 7 μm sections cut. Haematoxylin and eosin (H&E) staining, *in situ* hybridisation and periodic acid Schiff staining for glycogen were performed as described previously (Tunster et al., 2010).

Gene expression analysis

Quantitative PCR of reverse transcribed RNA was performed on n = 4 per genotype, with litter matched controls (n = 2+2 per litter) as described previously (Tunster et al., 2010).

Placental glycogen measurement

Glycogen was extracted from whole placenta as described previously, resuspended in 1 ml of H_2O, diluted 1 in 2, and glycogen concentration determined using the phenol-sulphuric acid method (Lo et al., 1970).

Statistical analyses

The χ^2 test was performed to determine whether the number of conceptuses observed differed from the expected frequency for each genotype. A one-way ANOVA in conjunction with the Bonferroni correction was used to compare fetal and placental weights and placental glycogen content between genotypes. Statistical significance for analysis of gene expression was determined using the Student's t-Test (two tailed distribution and two sample unequal variance) (Schmittgen and Livak, 2008).

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Competing Interests

The authors declare that there is no conflict of interest financial or otherwise associated with this submission.

Author Contributions

RMJ and SJT conceived and designed the experiments, interpreted the data and wrote the paper. SJT performed most of the experimental work, supported by MVdP. HDJC supported image capture and analysis. LL developed and provided the DelTel7 mouse line. SJT, RMJ and LL wrote and revised the manuscript. All authors read and approved the final manuscript.

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Tables

E14.5			
	WT	***Ascl2*^{BAC}**	**DelTel7^{BAC}**
Observed	61	62	35
Expected	52.67	52.67	52.67
% of expected	116%	118%	66%
χ^2	8.90 > 5.991		
E18.5			
	WT	***Ascl2*^{BAC}**	**DelTel7^{BAC}**
Observed	66	51	30
Expected	46.67	46.67	46.67
% of expected	135%	104%	61%
χ^2	13.35 > 5.991		

Table 1: Restoring *Ascl2* gene expression rescues embryonic lethality in approximately two-thirds of DelTel7 conceptuses. Male *Ascl2*^{BAC} mice were mated with female DelTel7 mice to generate 22 litters at E14.5 and 26 litters at E18.5. DelTel^{BAC} conceptuses were recovered at approximately 60% of the expected frequency.

E18.5 *Cdkn1c*$^{+/-}$ x *Ascl2*^{BAC}				
	WT	***Ascl2*^{BAC}**	***Cdkn1c*$^{-/+}$**	***Cdkn1c*$^{-/+BAC}$**
Observed	35	42	46	27
Expected	37.5	37.5	37.5	37.5
χ^2	5.57 < 7.815			
E18.5 *Phlda2*$^{+/-}$ x *Ascl2*^{BAC}				
	WT	***Ascl2*^{BAC}**	***Phlda2*$^{-/+}$**	***Phlda2*$^{-/+BAC}$**
Observed	54	38	51	44
Expected	46.75	46.75	46.75	46.75
χ^2	3.31 < 7.815			

Table 2: *Ascl2*^{BAC} does not cause embryonic lethality on *Cdkn1c* or *Phlda2* null backgrounds. Male *Ascl2*^{BAC} mice were mated with female mice carrying either a *Cdkn1c* or *Phlda2* targeted allele, generating a total of 46 litters at E18.5. 20 litters (150 embryos) were generated from females carrying the *Cdkn1c* targeted allele and 26 litters (187 embryos) from females carrying the *Phlda2* targeted allele. There was no significant difference between the observed and expected genotype frequency.

Figures

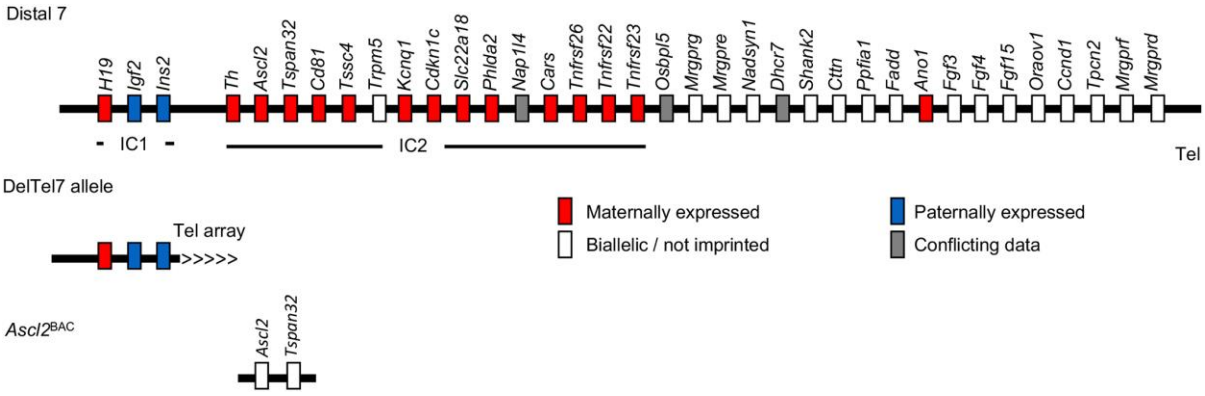

Figure 1: Schematic of DelTel7 and *Ascl2*^{BAC} models. Schematic of mouse distal chromosome 7 showing the extent of the DelTel7 deletion, which encompasses the entire IC2 domain and ~20 telomeric genes, whilst leaving the IC1 domain intact. Imprinting status from (Tunster et al., 2013).

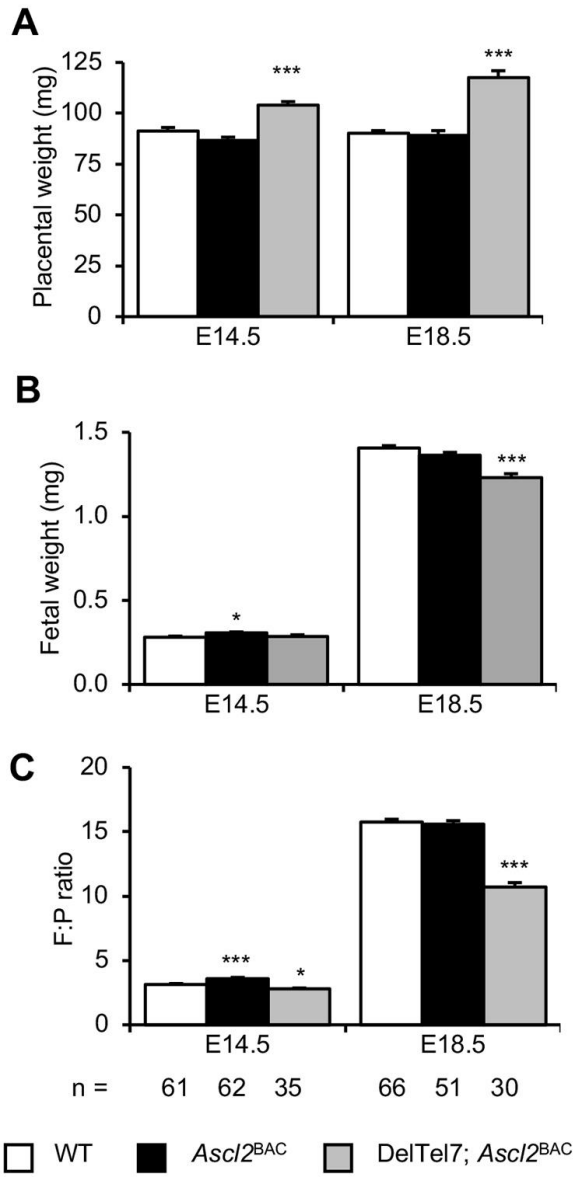

Figure 2: Restoring *Ascl2* gene function rescues DelTel7 lethality. (A) Placental wet weights at E14.5 and E18.5 for the three viable genotypes generated from mating *Ascl2*^{BAC} (129) males with DelTel7 (CD1) females. *Ascl2*^{BAC} placentae were not significantly different in weight relative to controls at either stage. DelTel7^{BAC} placentae were significantly heavier than control littermates at both E14.5 and E18.5. (B) Corresponding fetal wet weights at E14.5 and E18.5. Although *Ascl2*^{BAC} fetuses were slightly heavier than control littermates at E14.5, fetal weight was normalised at E18.5. In contrast, whilst DelTel7^{BAC} fetuses were no different in weight to controls at E14.5, they were substantially growth restricted at E18.5. (C) Fetal:placental (F:P) ratios at E14.5 and E18.5. Placental efficiency was slightly increased for *Ascl2*^{BAC} at E14.5 but normalised at E18.5. Efficiency of DelTel7^{BAC} placentae was reduced at both E14.5 and E18.5. Numerical data is given in Supplementary Table S1. * $p < 0.05$, *** $p < 0.005$.

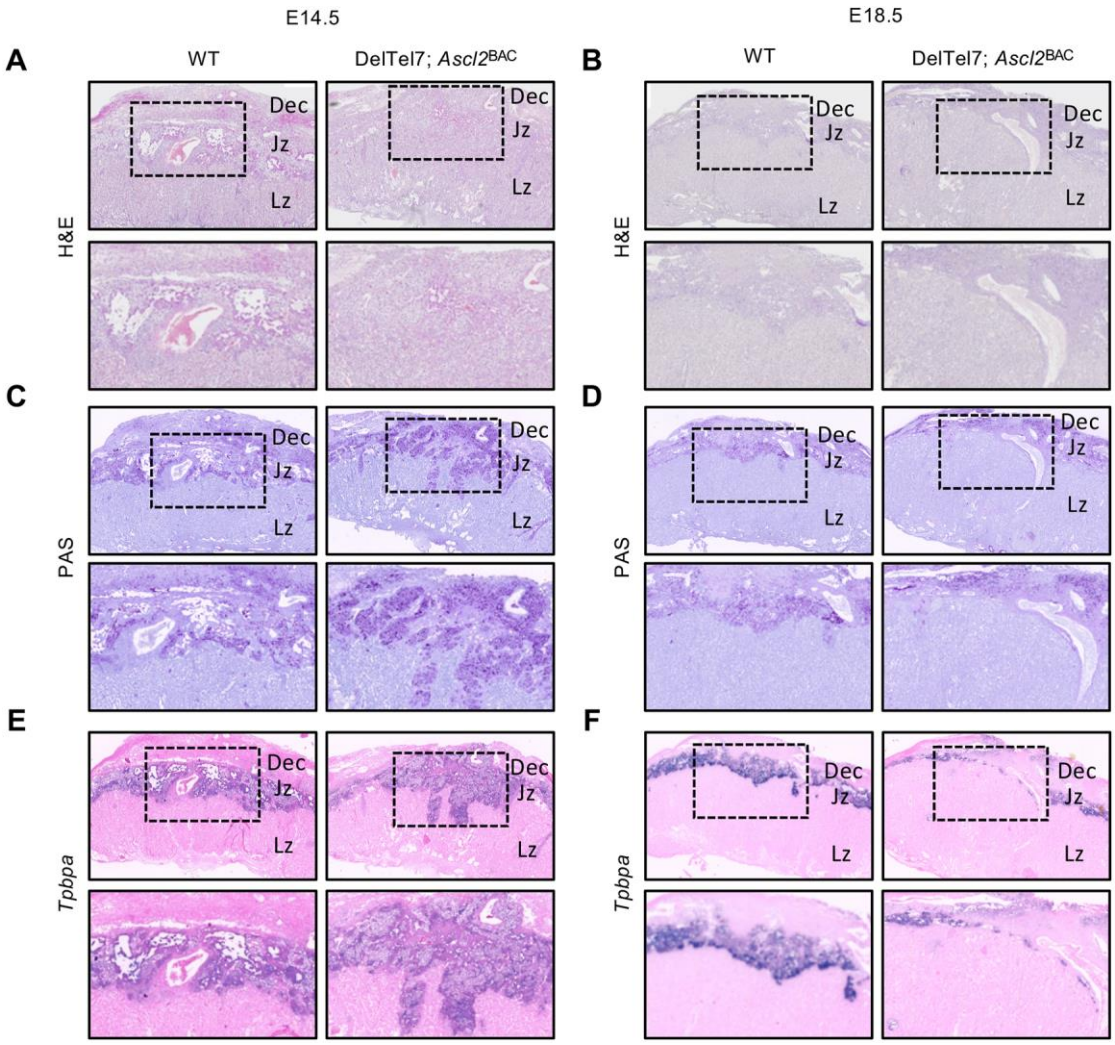

Figure 3: Disrupted and diminished junctional zone. A and B) Haematoxylin and Eosin staining of midline placental sections of control (left) and DelTel7^{BAC} (right) placentae at E14.5 (A) and E18.5 (B). Higher magnification of boxed area shown below. C and D) Periodic Acid Schiff staining of adjacent sections demonstrating mislocalisation of GlyT at E14.5 (C) and diminished glycogen staining at E18.5 (D). Higher magnification of boxed area shown below. E and F) *In situ* hybridisation with a probe for the Jz marker *Tpbpa* further demonstrating the Jz mislocalisation defect at E14.5 (E) and diminished Jz at E18.5 (F). Higher magnification of boxed area shown below.

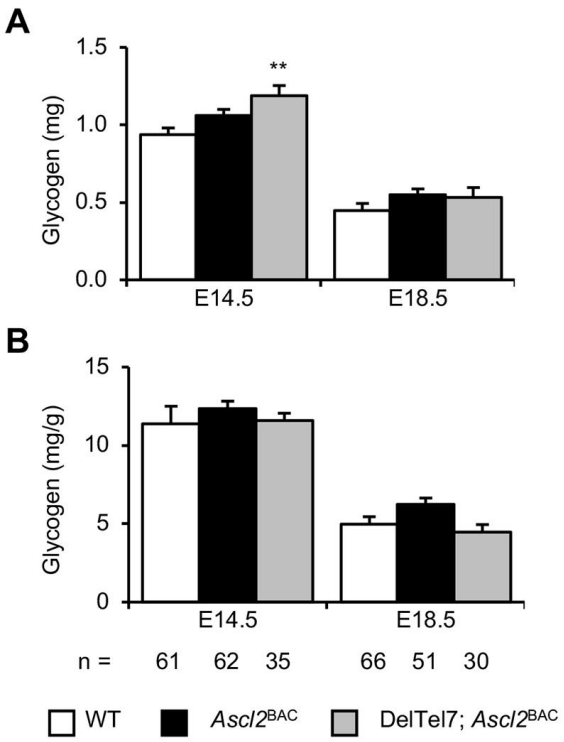

Figure 4: Transient increase in placental glycogen. (A) Total placental glycogen of control, *Ascl2*^{BAC} and DelTel7^{BAC} placentae at E14.5 and E18.5. Total placental glycogen stores of DelTel7^{BAC} were ~25% greater than controls at E14.5, but unaltered at E18.5. (B) When normalise by placental weight, DelTel7^{BAC} placental glycogen stores expressed as mg glycogen per g of placenta were unaltered. Numerical data is given in Supplementary Table S2. ** $p < 0.01$.

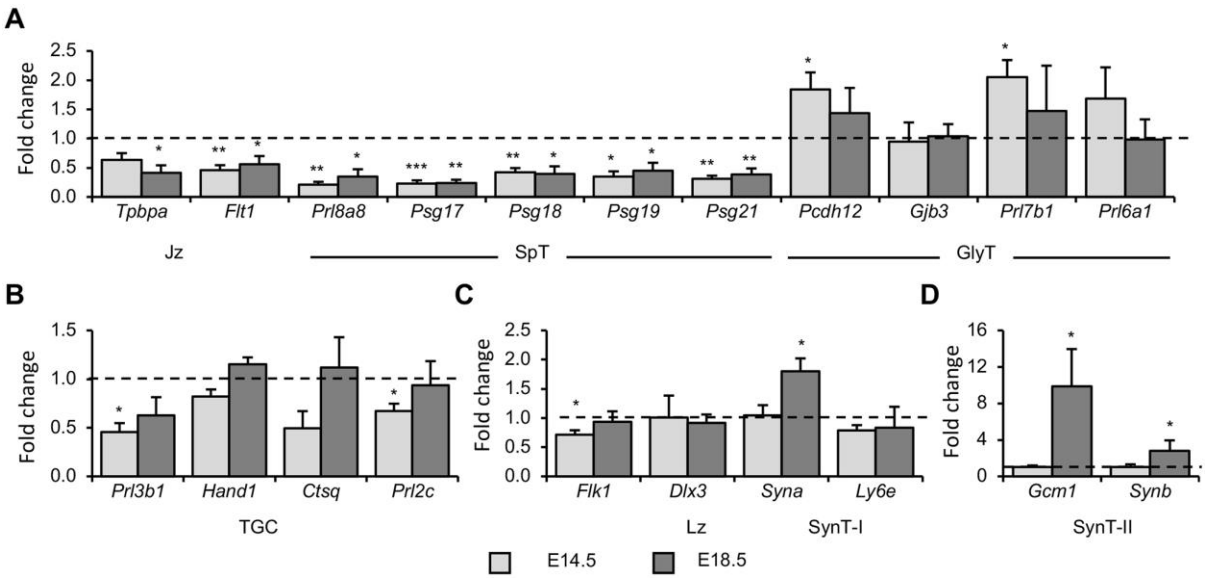

Figure 5: Relative expression levels of key trophoblast lineage markers. (A) Expression of Junctional zone and SpT markers were similarly reduced at E14.5 and E18.5, whereas expression of GlyT markers was either slightly elevated or unaltered at these stages. (B) No consistent alteration in expression of TGC markers was observed, with *Prl3b1* and *Prl2c* reduced at E14.5 but not at E18.5, whilst expression of *Hand1* and *Ctsq* was not significantly altered at either stage. (C) *Flk1*, which is predominantly expressed in the fetal vasculature of the labyrinth, was reduced at E14.5 but unaltered at E18.5. Expression of *Dlx3*, which is widely expressed in the labyrinth, was unaltered at both stages. Expression of *Syna* and *Ly6e*, which are both expressed in SynT-I, were unaltered at E14.5, with *Syna* was elevated ~80% at E18.5, although *Ly6e* remained unaltered at this stage. (D) Expression of the SynT-II markers *Gcm1* and *Synb* was unaltered at E14.5, but dramatically increased at E18.5, with *Gcm1* elevated ~10-fold and *Synb* elevated ~4-fold. N = 4 v 4 from at least 2 litters. Numerical data is given in Supplementary Table S3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

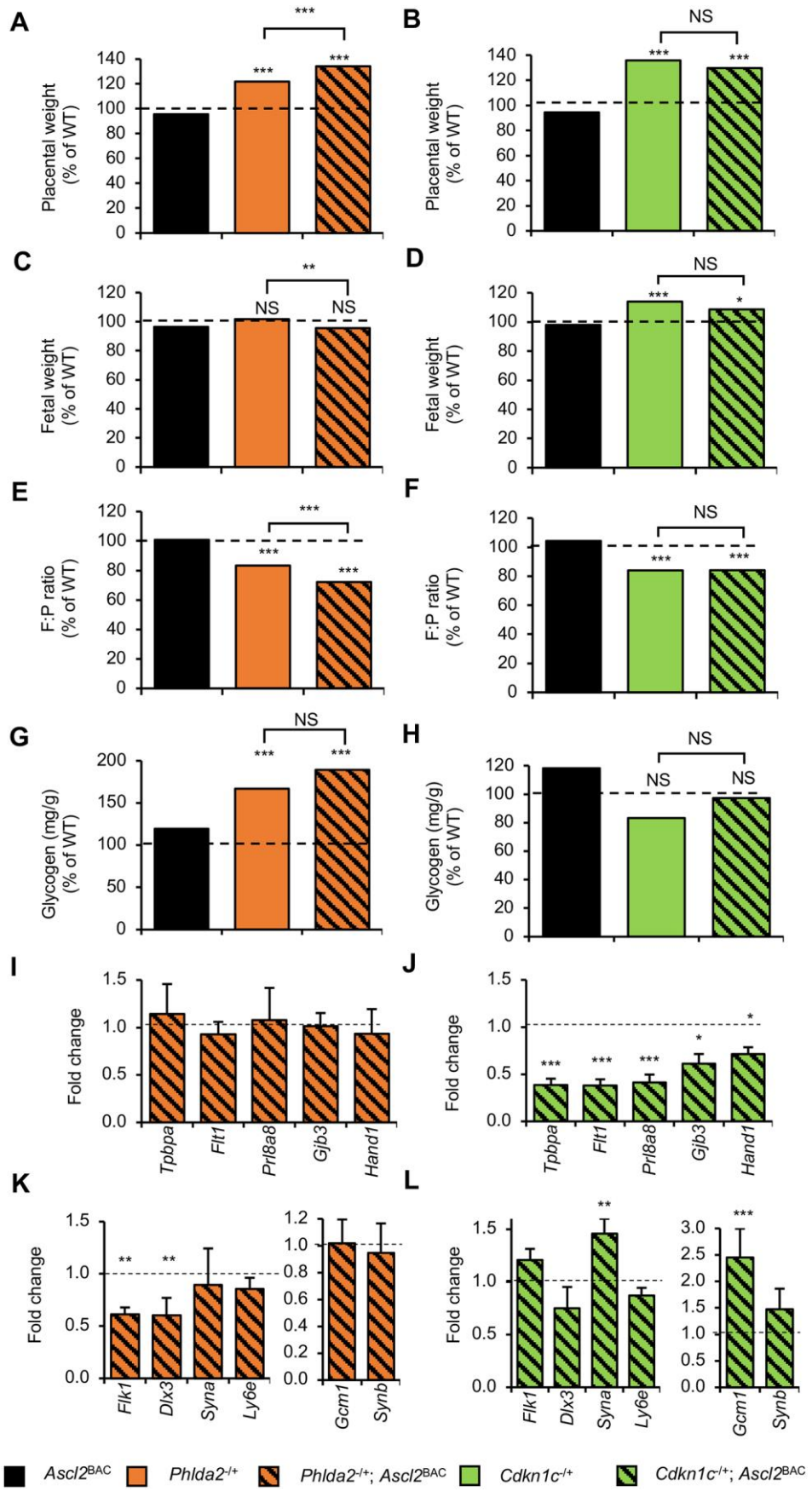

Figure 6: Excluding a contribution of elevated *Ascl2*. To exclude a contribution of elevated *Ascl2* in the observed phenotypes we investigated the phenotypic outcomes associated with *Ascl2*^{BAC} combined with loss of function of either *Phlda2* or *Cdkn1c* in isolation at E18.5. Placental weights from matings between *Ascl2*^{BAC} males and females carrying either a *Phlda2* (A) or *Cdkn1c* (B) loss of function allele. Consistent with previous reports, both *Phlda2*$^{-/+}$ and *Cdkn1c*$^{-/+}$ placentae were substantially heavier than control littermates. Elevated expression of *Ascl2*^{BAC} did not affect placental overgrowth of *Cdkn1c*$^{-/+}$, with *Phlda2*$^{-/+BAC}$ placentae significantly heavier than both control and *Phlda2*$^{-/+}$. (C) *Phlda2*$^{-/+}$ and *Phlda2*$^{-/+BAC}$ fetuses did not differ in weight compared to control littermates, although *Phlda2*$^{-/+BAC}$ fetuses were slightly lighter than *Phlda2*$^{-/+}$. (D) *Cdkn1c*$^{-/+}$ and *Cdkn1c*$^{-/+BAC}$ fetuses were substantially heavier than control littermates, with no significant difference in fetal weight between *Cdkn1c*$^{-/+}$ and *Cdkn1c*$^{-/+BAC}$. (E, F) The substantial placentomegaly coupled with the absence of any negative effect on fetal growth resulted in substantially diminished measures of placental efficiency (F:P ratio) for all genotypes except *Ascl2*^{BAC}. For A, C, E; n = WT: 54, *Ascl2*^{BAC}: 38, *Phlda2*$^{-/+}$: 51, *Phlda2*$^{-/+BAC}$: 44; For B, D, F; n = WT: 35, *Ascl2*^{BAC}: 42, *Cdkn1c*$^{-/+}$: 46, *Cdkn1c*$^{-/+BAC}$: 27. For G; n = WT: 47, *Ascl2*^{BAC}: 29, *Phlda2*$^{-/+}$: 36, *Phlda2*$^{-/+BAC}$: 32; For H; n = WT: 40, *Ascl2*^{BAC}: 44, *Cdkn1c*$^{-/+}$: 38, *Cdkn1c*$^{-/+BAC}$: 22. For I, J, K, L, n = 4 v 4 from at least 2 litters. Numerical data is given in Supplementary Table S4. NS $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Table S1: Data for Figure 2

A: Placental weight (mg)		
	E14.5	**E18.5**
WT	91.4 mg ± 1.6 n = 61	90.2 mg ± 1.2 n = 66
***Ascl2*^{BAC}**	86.7 mg ± 1.6 n = 62	89.3 mg ± 2.2 n = 51
DelTel7^{BAC}	104.0 mg ± 1.7 n = 35	117.5 mg ± 3.4 n = 30
ANOVA	$F_{2,155}$ = 23.4, p = 1.27 x 10^{-9}	$F_{2,144}$ = 46.2, p = 3.10 x 10^{-16}
Ratio and Bonferroni corrected *p* value: WT vs *Ascl2*^{BAC}	94.8% p = 0.0930	99.0% p = 1.000
Ratio and Bonferroni corrected *p* value: WT vs DelTel7^{BAC}	113.8% p = 6.21 x 10^{-6}	130.3% p = 1.16 x 10^{-14}
B: Fetal weight (g)		
WT	0.282 g ± 0.005 n = 61	1.405 g ± 0.015 n = 66
***Ascl2*^{BAC}**	0.306 g ± 0.006 n = 62	1.364 g ± 0.016 n = 51
DelTel7^{BAC}	0.287 g ± 0.008 n = 35	1.231 g ± 0.023 n = 30
ANOVA	$F_{2,155}$ = 4.5, p = 0.0124	$F_{2,144}$ = 22.0, p = 4.48 x 10^{-9}
Ratio and Bonferroni corrected *p* value: WT vs *Ascl2*^{BAC}	108.3% p = 0.0128	97.1% p = 0.206
Ratio and Bonferroni corrected *p* value: WT vs DelTel7^{BAC}	101.8% p = 1.000	87.6% p = 2.13 x 10^{-9}
C: F:P ratio		
WT	3.1 ± 0.07 n = 61	15.7 ± 0.24 n = 66
***Ascl2*^{BAC}**	3.6 ± 0.09 n = 62	15.6 ± 0.29 n = 51
DelTel7^{BAC}	2.8 ± 0.08 n = 35	10.7 ± 0.34 n = 30
ANOVA	$F_{2,155}$ = 21.2, p = 7.12 x 10^{-9}	$F_{2,144}$ = 76.6, p = 2.24 x 10^{-23}
Ratio and Bonferroni corrected *p* value: WT vs *Ascl2*^{BAC}	114.4% p = 1.76 x 10^{-4}	98.9% p = 1.000
Ratio and Bonferroni corrected *p* value: WT vs DelTel7^{BAC}	88.6% p = 0.0184	68.0% p = 4.60 x 10^{-22}

Table S2: Data for Figure 4

A: Glycogen (mg)		
	E14.5	**E18.5**
WT	0.94 mg ± 0.039 n = 61	0.45 mg ± 0.044 n = 60
***Ascl2*^{BAC}**	1.06 mg ± 0.052 n = 58	0.55 mg ± 0.037 n = 49
DelTel7^{BAC}	1.19 mg ± 0.048 n = 30	0.53 mg ± 0.065 n = 27
ANOVA	$F_{2,146}$ = 6.1, p = 0.00293	$F_{2,133}$ = 1.5, p = 0.224
Ratio and Bonferroni corrected *p* value: WT vs *Ascl2*^{BAC}	113.5% p = 0.122	122.4% p = 0.224
Ratio and Bonferroni corrected *p* value: WT vs DelTel7^{BAC}	127.0% p = 0.00261	118.3% p = 0.791
B: Glycogen (mg/g)		
WT	11.37 mg/g ± 1.13 n = 61	4.97 mg/g ± 0.47 n = 60
***Ascl2*^{BAC}**	12.34 mg/g ± 0.48 n = 58	6.22 mg/g ± 0.42 n = 49
DelTel7^{BAC}	11.57 mg/g ± 0.49 n = 30	4.45 mg/g ± 0.50 n = 27
ANOVA	$F_{2,146}$ = 0.39, p = 0.679	$F_{2,133}$ = 3.24, p = 0.0422
Ratio and Bonferroni corrected *p* value: WT vs *Ascl2*^{BAC}	108.5% p = 1.000	125.1% p = 0.140
Ratio and Bonferroni corrected *p* value: WT vs DelTel7^{BAC}	101.7% p = 1.000	89.5% p = 1.000

Table S3: Data for Figure 5

A: Jz markers			B: TGC markers		
	E14.5	**E18.5**		**E14.5**	**E18.5**
Tpbpa	0.64 ± 0.11 $p = 0.0557$	0.41 ± 0.13 $p = 0.0232$	***Prl3b1***	0.45 ± 0.09 $p = 0.0261$	0.63 ± 0.19 $p = 0.161$
Flt1	0.46 ± 0.08 $p = 1.15 \times 10^{-3}$	0.56 ± 0.14 $p = 0.0220$	***Hand1***	0.82 ± 0.07 $p = 0.0592$	1.15 ± 0.07 $p = 0.0637$
Prl8a8	0.21 ± 0.05 $p = 6.83 \times 10^{-3}$	0.35 ± 0.13 $p = 0.0122$	***Ctsq***	0.50 ± 0.17 $p = 0.140$	1.12 ± 0.31 $p = 0.715$
Psg17	0.23 ± 0.05 $p = 8.64 \times 10^{-5}$	0.24 ± 0.06 $p = 0.00366$	***Prl2c***	0.67 ± 0.08 $p = 0.0186$	0.93 ± 0.25 $p = 0.803$
Psg18	0.42 ± 0.08 $p = 4.87 \times 10^{-3}$	0.39 ± 0.13 $p = 0.0171$	**C: Lz and SynT-I markers**		
Psg19	0.35 ± 0.09 $p = 0.0182$	0.45 ± 0.13 $p = 0.0340$	***Flk1***	0.71 ± 0.08 $p = 0.0161$	0.93 ± 0.18 $p = 0.729$
Psg21	0.31 ± 0.05 $p = 0.00191$	0.39 ± 0.10 $p = 0.00423$	***Dlx3***	1.01 ± 0.37 $p = 0.980$	0.92 ± 0.14 $p = 0.612$
Pcdh12	1.84 ± 0.29 $p = 0.0138$	1.44 ± 0.43 $p = 0.281$	***Syna***	1.05 ± 0.09 $p = 0.592$	1.80 ± 0.36 $p = 0.0230$
Gjb3	0.95 ± 0.33 $p = 0.883$	1.04 ± 0.21 $p = 0.843$	***Ly6e***	0.79 ± 0.14 $p = 0.209$	0.83 ± 0.11 $p = 0.256$
Prl7b1	2.05 ± 0.30 $p = 0.0107$	1.48 ± 0.77 $p = 0.547$	**B: SynT-II markers**		
Prl6a1	1.69 ± 0.53 $p = 0.139$	0.98 ± 0.35 $p = 0.956$	***Gcm1***	1.05 ± 0.15 $p = 0.767$	9.87 ± 4.08 $p = 0.0212$
			Synb	1.03 ± 0.29 $p = 0.927$	2.82 ± 1.13 $p = 0.0280$

Table S4: Data for Figure 6

A: Placental weight (mg)			
	E18.5	**Ratio and Bonferroni corrected *p* value vs WT**	**Ratio and Bonferroni corrected *p* value:**
WT	87.7 mg ± 1.28 n = 54	-	-
***Ascl2*^{BAC}**	83.8 mg ± 1.71 n = 38	95.6% $p = 0.505$	-
***Phlda2*$^{-/+}$**	106.9 mg ± 1.53 n = 51	121.9% $p = 6.73 \times 10^{-12}$	**vs *Phlda2*$^{-/+}$**
***Phlda2*$^{-/+BAC}$**	117.0 mg ± 3.00 n = 44	134.2% $p = 1.00 \times 10^{-16}$	110.1% $p = 1.82 \times 10^{-4}$
ANOVA	$F_{3,183} = 66.2$, $p = 1.11 \times 10^{-16}$		
B: Placental weight (mg)			
	E18.5	**Ratio and Bonferroni corrected *p* value vs WT**	**Ratio and Bonferroni corrected *p* value:**
WT	84.0 mg ± 1.44 n = 35	-	-
***Ascl2*^{BAC}**	79.3 mg ± 1.52 n = 42	94.4% $p = 0.0495$	-
***Cdkn1c*$^{-/+}$**	114.1 mg ± 1.58 n = 46	135.8% $p = 1.00 \times 10^{-20}$	**vs *Cdkn1c*$^{-/+}$**
***Cdkn1c*$^{-/+BAC}$**	109.0 mg ± 2.44 n = 27	129.7% $p = 6.66 \times 10^{-16}$	95.5% $p = 0.0946$
ANOVA	$F_{3,146} = 111.5$, $p = 1.11 \times 10^{-16}$		
C: Fetal weight (g)			
WT	1.29 g ± 0.014 n = 54	-	-
***Ascl2*^{BAC}**	1.24 g ± 0.021 n = 38	96.4% $p = 0.154$	-
***Phlda2*$^{-/+}$**	1.31 g ± 0.015 n = 51	101.7% $p = 0.604$	**vs *Phlda2*$^{-/+}$**
***Phlda2*$^{-/+BAC}$**	1.22 g ± 0.017 n = 44	95.6% $p = 0.0752$	93.9% $p = 0.00416$
ANOVA	$F_{3,183} = 5.29$, $p = 1.60 \times 10^{-3}$		
D: Fetal weight (g)			
WT	1.29 g ± 0.023 n = 35	-	-
***Ascl2*^{BAC}**	1.27 g ± 0.021 n = 42	98.0% $p = 0.473$	-
***Cdkn1c*$^{-/+}$**	1.47 g ± 0.028 n = 46	114.0% $p = 6.21 \times 10^{-6}$	**vs *Cdkn1c*$^{-/+}$**
***Cdkn1c*$^{-/+BAC}$**	1.40 g ± 0.031 n = 27	108.6% $p = 0.0232$	95.3% $p = 0.155$
ANOVA	$F_{3,146} = 15.4$, $p = 9.92 \times 10^{-9}$		

E: F:P ratio			
WT	14.8 ± 0.25 n = 54	-	-
***Ascl2*^{BAC}**	14.9 ± 0.30 n = 38	100.8% $p = 0.760$	-
***Phlda2*$^{-/+}$**	12.4 ± 0.20 n = 51	83.3% $p = 3.20 \times 10^{-11}$	**vs *Phlda2*$^{-/+}$**
***Phlda2*$^{-/+BAC}$**	10.7 ± 0.27 n = 44	72.2% $p = 1.00 \times 10^{-26}$	86.6% $p = 1.57 \times 10^{-5}$
ANOVA	$F_{3,183} = 62.5$, $p = 1.11 \times 10^{-16}$		
F: F:P ratio			
WT	15.5 ± 0.35 n = 35	-	-
***Ascl2*^{BAC}**	16.2 ± 0.38 n = 42	104.3% $p = 0.369$	-
***Cdkn1c*$^{-/+}$**	13.0 ± 0.31 n = 46	84.0% $p = 6.59 \times 10^{-6}$	**vs *Cdkn1c*$^{-/+}$**
***Cdkn1c*$^{-/+BAC}$**	13.0 ± 0.42 n = 27	84.1% $p = 8.95 \times 10^{-5}$	100.1% $p = 0.984$
ANOVA	$F_{3,146} = 21.1$, $p = 2.14 \times 10^{-11}$		
G: Glycogen (mg/g)			
WT	5.42 ± 0.26 n = 47	-	-
***Ascl2*^{BAC}**	6.47 ± 0.44 n = 29	119.4% $p = 0.0719$	-
***Phlda2*$^{-/+}$**	9.04 ± 0.36 n = 36	166.9% $p = 2.93 \times 10^{-9}$	**vs *Phlda2*$^{-/+}$**
***Phlda2*$^{-/+BAC}$**	10.25 ± 0.63 n = 32	189.2% $p = 9.46 \times 10^{-14}$	113.4% $P = 0.266$
ANOVA	$F_{3,140} = 30.9$, $p = 2.11 \times 10^{-15}$		
H: Glycogen (mg/g)			
WT	4.56 ± 0.38 n = 40	-	-
***Ascl2*^{BAC}**	5.39 ± 0.37 n = 44	118.2% $p = 0.409$	-
***Cdkn1c*$^{-/+}$**	3.80 ± 0.24 n = 38	83.2% $p = 0.363$	**vs *Cdkn1c*$^{-/+}$**
***Cdkn1c*$^{-/+BAC}$**	4.44 ± 0.46 n = 22	97.3% $p = 0.828$	116.9% $p = 0.543$
ANOVA	$F_{3,140} = 3.75$, $p = 0.0125$		
I & J: Jz Markers			
	***Phlda2*$^{-/+BAC}$**	***Cdkn1c*$^{-/+BAC}$**	
Tpbpa	1.14 ± 0.31 $p = 0.680$	0.38 ± 0.07 $p = 0.00316$	
Flt1	0.93 ± 0.13 $p = 0.617$	0.38 ± 0.06 $p = 1.07 \times 10^{-4}$	
Prl8a8	1.08 ± 0.34 $p = 0.821$	0.41 ± 0.08 $p = 0.00726$	
Gjb3	1.02 ± 0.14	0.61 ± 0.10	

	$p = 0.913$	$p = 0.0299$
Hand1	0.93 ± 0.26 $p = 0.857$	0.71 ± 0.07 $p = 0.0135$
K & L: Lz markers		
Flk1	0.61 ± 0.06 $p = 0.0013$	1.21 ± 0.11 $p = 0.0948$
Dlx3	0.61 ± 0.16 $p = 0.0607$	0.75 ± 0.20 $p = 0.282$
Syna	0.89 ± 0.35 $p = 0.789$	1.46 ± 0.14 $p = 0.00600$
Ly6e	0.85 ± 0.11 $p = 0.279$	0.87 ± 0.07 $p = 0.155$
Gcm1	1.02 ± 0.18 $p = 0.924$	2.45 ± 0.54 $p = 0.00300$
Synb	0.95 ± 0.22 $p = 0.819$	1.47 ± 0.39 $p = 0.194$