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Loss of *Cited2* affects trophoblast formation and vascularization of the mouse placenta

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Abstract

Cited2 is widely expressed in the developing embryo and in extraembryonic tissues including the placenta. Gene expression can be induced by a number of factors; most notably by the hypoxia inducible transcription factor, HIF1, under low oxygen conditions. *Cited2* encodes for a transcriptional co-factor that in vitro can act as both a positive and negative regulator of transcription. This function is due to its interaction with CBP/p300 and appears to depend on whether Cited2 enables CBP/p300 to interact with the basic transcriptional machinery, or if its binding prevents such an interaction from occurring. Here, we report a novel function for *Cited2* in placenta formation, following gene deletion in mouse. In the absence of *Cited2* the placenta and embryo are significantly small from 12.5 and 14.5 dpc respectively, and death occurs in utero. *Cited2* null placentas have fewer differentiated trophoblast cell types; specifically there is a reduction in trophoblast giant cells, spongiotrophoblasts and glycogen cells. In addition, the fetal vasculature of the placenta is disorganised and there are fewer anastomosing capillaries. Given that *Cited2* is expressed in both trophoblasts and the fetal vasculature, the observed defects fit well with the sites of gene expression. We conclude that *Cited2* is required for normal placental development and vascularisation, and hence for embryo viability.

Keywords: Cited2; Placenta; Mouse; Vasculature; Trophoblast; Gene targeting

Introduction

The placenta is essential for growth and development of the embryo and, although it functions only during gestation, the effects of poor function in utero can manifest well into adult life (Barker, 2004). Efficient functioning of the placenta requires that the key cell types are arranged into recognized layers, and that they are present in sufficient number. The mouse placenta consists of distinct layers that are defined according to their relative position within the placenta, and by their resident cell types. The fetal layers of the placenta are proximal to the fetus and include the labyrinthine, spongiotrophoblast (also known as the junctional zone), and trophoblast giant cell (TGC) layers. Each of these layers consist of trophoblasts, and the labyrinthine layer also contains fetal endothelial cells. Proximal to the mother is the maternal decidua, which consists of maternal endothelial and decidual cells, and trophoblasts that have migrated in from the fetal placenta. Trophoblast stem (TS) cells are located in the extraembryonic ectoderm, and later, the chorionic ectoderm (Tanaka et al., 1998; Uy et al., 2002). They are capable of differentiating into all trophoblast cell types of the placenta, and provide the overlying ectoplacental cone (EPC) with progenitors for both spongiotrophoblasts and secondary TGC

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(Carney et al., 1993; Johnson and Rossant, 1981; Rossant et al., 1978; Simmons and Cross, 2005).

Although the different layers of the placenta are already formed by 10.5 dpc, they continue to grow and elaborate to meet the demands of the embryo, and undergo remodelling facilitated by the release of matrix metalloproteinases (Teesalu et al., 1999). Regulating proliferation of progenitor cells and their differentiation into the various trophoblast cell types are clearly crucial for development of a normal placenta, and the balance between these processes needs to be tightly controlled. Growth factors, transcription factors and oxygen, influence proliferation and differentiation of trophoblast and endothelial cells (Red-Horse et al., 2004; Simmons and Cross, 2005). The molecular complexity required to control placental development and function is apparent, as mutation of increasing numbers of genes result in placental insufficiency and fetal demise (Watson and Cross, 2005).

Expression of the transcriptional modulator *Cited2* can be induced by various stimuli including hypoxia, cytokines, growth factors, lipopolysaccharide and shear flow (Bhattacharya et al., 1999; Sun et al., 1998; Yokota et al., 2003). *Cited2* expression is also upregulated by progesterone signalling (Jeong et al., 2005), in common with the transcription factor hypoxia inducible factor 1α (HIF1α; Daikoku et al., 2003). HIF1α and Cited2 interact with CBP and p300, homologous proteins that have acetyltransferase and E4 ubiquitin ligase activities (Chan and La Thangue, 2001; Grossman et al., 2003). HIF1α requires CBP/p300 in order to activate target gene transcription, and since HIF1α and Cited2 bind overlapping sites on CBP/p300, it is postulated that Cited2 acts as a negative regulator of HIF1α activity (De Guzman et al., 2004; Freedman et al., 2003). Cited2 also appears to displace Ets1 from CBP/p300 (Yokota et al., 2003), suggesting that Cited2 may also function to inhibit Ets1 activity. In contrast, it has been shown that Cited2 can bind directly to DNA binding proteins in vitro (Lhx2, TFAP2, PPARα, PPARγ), and through the recruitment of CBP/p300 acts as a positive regulator of transcription (Bamforth et al., 2001; Glenn and Maurer, 1999; Tien et al., 2004). Deletion of *HIF1α*/*Arnt*, *PPARγ* or *TFAP2γ* all produce placental phenotypes (Adelman et al., 1999, 2000; Auman et al., 2002; Barak et al., 1999; Kozak et al., 1997; Maltepe et al., 1997). Therefore, *Cited2* has the potential to influence placental development at many levels. In addition, loss of *Cited2* results in fibroblast senescence (Kranc et al., 2003), and overexpression of *Cited2* results in oncogenic cell transformation (Sun et al., 1998). Such responses suggest that *Cited2* may function in cell growth control (Sun et al., 1998).

Cited2 is widely expressed in both embryonic and extra-embryonic cells during early development (Dunwoodie et al., 1998; Martinez-Barbera et al., 2002; Weninger et al., 2005). *Cited2* is essential for embryonic development; *Cited2* null embryos die during gestation with abnormal development of numerous organs. They display a diverse array of cardiac malformations, and show defects in establishment of the left–right body axis, neural and adrenal development (Bamforth et al., 2001, 2004; Martinez-Barbera et al., 2002; Schneider et al., 2003; Weninger and Mohun, 2002; Weninger et al., 2005; Yin et al., 2002). Hypoxia-activated gene transcription is also apparently deregulated in *Cited2* null embryos (Yin et al., 2002).

We report here that *Cited2* nulls also display profound defects in placental development. Total placenta size is greatly reduced, with the secondary TGC, spongiotrophoblasts, and trophoblasts in the decidua affected in particular. These layers correlate with domains of strongest *Cited2* expression. *Cited2* is not expressed in TS cells, but onset of expression is coincident with differentiation into TGC or spongiotrophoblasts in vitro. We therefore propose that *Cited2* acts to regulate the production of TGC and spongiotrophoblasts during placental development. Unusually, *Cited2* is expressed in both the mesoderm-derived fetal vessels that penetrate into the placenta, as well as in trophoblast cells. Integrity of the fetal vessel walls and capillary formation are clearly compromised in *Cited2* mutant placentas, indicating that *Cited2* also plays a direct role in vascular development. Therefore we provide evidence that *Cited2* functions in several different cell types during placental development.

Materials and methods

Mouse lines and genotyping

The *Cited2-lacZ* mouse line (*Cited2*^{tm1Jpmb}; (Martinez-Barbera et al., 2002) was maintained on a C57BL/6:129 hybrid genetic background following four backcrosses onto C57BL/6. Mice heterozygous for this *Cited2* targeted allele *(Cited2*$^{+/-}$*)* were intercrossed to produce *Cited2* wildtype *(Cited2*$^{+/+}$*)*, heterozygous *(Cited2*$^{+/-}$*)* and homozygous null *(Cited2*$^{-/-}$*)* embryos for analysis. Mice and embryos were genotyped by PCR using primers as described previously (Martinez-Barbera et al., 2002). The *Cited1*^{neo} mouse line (*Cited1*^{tm1Dunw}; Rodriguez et al., 2004) was maintained on a C57BL/6 genetic background. Mice and embryos were genotyped by PCR using primers as described previously (Rodriguez et al., 2004). All of the double mutant embryos analysed in this paper were produced from crosses between *Cited1*$^{+/neo}$*/Cited2*$^{+/-}$ females and *Cited1*$^{neo/Y}$*/Cited2*$^{+/-}$ males. Noon of the day of appearance of the vaginal plug is designated 0.5 days *post coitum* (dpc). Mice were sacrificed by cervical dislocation.

Histological and gene expression analysis

Histology, RNA in situ hybridisation and X-gal staining were performed on placental sections as previously described (Dunwoodie et al., 1997; Hogan et al., 1994; Kaufman, 1992; Rodriguez et al., 2004). cDNA probe details are as follows: *Cited2* (Dunwoodie et al., 1998); *Tpbp/4311* (Carney et al., 1993); *proliferin* (*PLF*; Lee et al., 1988); mouse *placental lactogen II* (*mPLII*; Shida et al., 1992); and *Mest* (*peg1*; Mayer et al., 2000). Alkaline phosphatase activity was detected as previously described (Rodriguez et al., 2004).

Immunohistochemistry

Embryos dissected in phosphate buffered saline (PBS) were fixed for 1 h in 4% paraformaldehyde (PFA), dehydrated, embedded in paraffin, 4-μm sections were cut and mounted on Superfrost Plus adhesion slides (Lomb Scientific P/L). For histological analysis, sections were stained with hematoxylin and eosin. For detection of alpha smooth muscle actin (αSMA), antigen retrieval was performed by incubating sections (3 times) in boiled 10 mM citrate buffer for 5 min. Sections were incubated in 0.3% hydrogen peroxide (PBS) for 30 min, washed, and incubated in 2% blocking reagent (Boehringer #1096176) for 1 h. Antibody incubation was with mouse anti-human αSMA (Dako M0851) at 1:50 for 60 min, and then with biotinylated rabbit anti-mouse FAB fragments (Vector Labs) at 1:200 for 10 min. The ABC detection system (Vector Labs) was used with 3,3′-diaminobenzidine (DAB) as substrate. The following reagents were also used: anti-BrdU (1: 200 dilution; Dako), anti-cleaved (active) caspase-3 (1: 400 dilution; Cell Signalling), and anti-p53 CM5 (1: 100 dilution; Novocastra Laboratories).

Measurement of placental layers

RNA in situ hybridisation was performed on cryosectioned placentas using the *4311* probe. Digital photographs were taken of three central sections of each placenta. NIH Image software was used to measure the area of the relevant stained/unstained regions of each section. For each placental section, the area was measured of the whole placenta, the labyrinthine layer, spongiotrophoblast layer, glycogen cells, and maternal decidua layers (Table 1). Since the total size of all *Cited2* null placentas was reduced compared to control littermates, the area of each layer was also represented as a percentage of the total placental area.

Generation of resin vascular casts and scanning electron microscopy

Casts of both the fetal and maternal blood spaces in the placenta at 14.5 dpc were generated as described previously (Adamson et al., 2002; Rodriguez et al., 2004). Tissue surrounding the resin casts was digested away using 20–30% KOH in H_2O. The casts were then washed thoroughly with distilled water, air-dried, sputtered with gold and analysed with a scanning electron microscope (Cambridge S360).

BrdU incorporation

Pregnant females at 11.5, 12.5 and 14.5 dpc were injected with BrdU (100 μg/g body) into the peritoneum 3 h before sacrifice. Placentas were fixed in 4% PFA for 1 h, and processed for immunohistochemistry as above. The mitotic index was calculated as the percentage of total cells with BrdU-positive nuclei.

Identification of low oxygen containing cells of the placenta

Hypoxyprobe (pimonidazole hydrochloride kit 100MAb1, Chemicon; 20 mg/ml solution in 0.9% NaCl) was injected (60 mg/kg) into the peritoneum of a pregnant mouse at 10.5 and 14.5 dpc. After 3 h the mouse was sacrificed (without the use of carbon dioxide), and the placentas were dissected in 10% formalin. The placentas were fixed in 10% formalin for 2–16 h at room temperature, incubated twice in 70% ethanol and stored. For detection of low oxygen cells, immunostaining was performed as described (Raleigh et al., 1998) with the following modifications. Instead of incubating in streptavidin peroxidase, sections were incubated for 45 min at room temperature in the ABC reagent (Vector Labs).

TS cell cultures and in situ hybridisation on cultured cells

TS cells were cultured in 6-well plates according to standard methods (Tanaka et al., 1998). Once reasonably confluent, cultures were split 1:3 and seeded onto glass coverslips (in 6-well plates). Cells were cultured for 2 days in TS cell medium, then 2 days in differentiation medium (lacking FGF4 and heparin). Coverslips were processed for in situ hybridisation by fixing the cells in 4% PFA for 20 min, washing in PBS and digesting with proteinase K for 15 min at room temperature. After re-fixation, cells were treated with triethanolamine/acetic anhydride for 2 × 5 min. Probe was diluted 1:250–1:1000 in hybridisation buffer, and hybridisation was carried out overnight at 52°C. Washes were performed with an SSC series and RNA buffer (including RNase A treatment). Cells were blocked using NT (0.1 M Tris pH 7.5, 150 mM NaCl, in water) buffer/ 1% milk powder/ 10% goat serum for 60 min at 37°C. Anti-DIG FAB fragments were diluted 1:800 in NT buffer/ 1% milk powder/ 1% goat serum overnight. Transcript localisation was detected using BCIP/NBT (Sigma) in NT. Development of the colour reaction was closely monitored as it was often rather fast and longer incubation caused lots of background. For photography, coverslips were inverted onto a slide with PBS/20% glycerol (Myriam Hemberger, unpublished).

Results

Cited2 null embryos die mid-gestation and display defects in numerous organs

We have previously reported that embryos homozygous for a null mutation in which the entire *Cited2* protein-coding region is replaced by *lacZ* are not viable. Systematic analysis of litters from *Cited2* heterozygous intercrosses indicated that in the C57BL/6:129 genetic background, null embryos die from 14.5 dpc (Martinez-Barbera et al., 2002; Weninger et al., 2005). As documented by ourselves and by others with independent *Cited2* targeted mutations, defects were found in numerous organs including the heart, brain and adrenal glands (Bamforth et al., 2001, 2004; Martinez-Barbera et al., 2002; Weninger et al., 2005; Yin et al., 2002). Here we report additional defects that are suggestive of poor blood circulation and vessel integrity, as well as anaemia (Fig. 1). Circulating blood in the yolk sac was noticeably reduced in 27/88 null embryos (Figs. 1A, D, E, H). 49/88 null embryos showed full body oedema (Fig. 1G). Debris in the amniotic fluid was found in 5 nulls, suggesting vessel leakage. Three umbilical vessels were found in 3 nulls, and in another case the umbilical vein bifurcated near to the junction with the placenta.

Importantly, we have also identified defects in placental development in our *Cited2* nulls, and demonstrate here for the first time that the placenta is an organ that requires *Cited2* function for its normal formation. Placenta size was reduced in all *Cited2* null conceptuses dissected from 12.5 dpc onwards (Figs. 1A, B, E, F). At 14.5 dpc, when most of the analyses were performed, all *Cited2* null conceptuses had small placentas. In addition, there were defects in organ formation clearly visible at the time of dissection in some of these placentas. For example, the outer layer of the placenta (maternal decidua) was greatly reduced in 30/88 nulls, and radiation of the major fetal vessels into the placenta was abridged and highly disorganised in 14/88 *Cited2* nulls (Figs. 1B, F). These clear phenotypes hinted at the

Table 1
Quantification of placenta size and of specific placental regions at 14.5 dpc

Genotype	Area (mm^2) [a]				
	Placenta	Labyrinth (% placental area) [b]	Spongiotrophoblasts	Glycogen cells [c]	Decidua
Cited2+/+ ($n = 3$)	14.34 ± 0.43	7.76 ± 0.28 (54.11 ± 0.97)	3.43 ± 0.24	1.56 ± 0.08	2.99 ± 0.13
Cited2+/− ($n = 2$)	12.74 ± 0.08*	7.52 ± 0.06 (59.03 ± 0.36)	2.35 ± 0.06**	1.33 ± 0.10	2.62 ± 0.08
Cited2−/− ($n = 5$)	9.99 ± 0.30**	5.87 ± 0.14** (58.76 ± 1.60)*	1.28 ± 0.12**	0.93 ± 0.06**	2.59 ± 0.13

Cited2+/+ was compared with *Cited2*+/−, and *Cited2*−/− using Student's *t* test, * ($P < 0.05$), ** ($P < 0.0001$).
[a] Mean ± standard error of mean.
[b] Represents proportion of placenta devoted to exchange.
[c] Glycogen cells in the decidua.

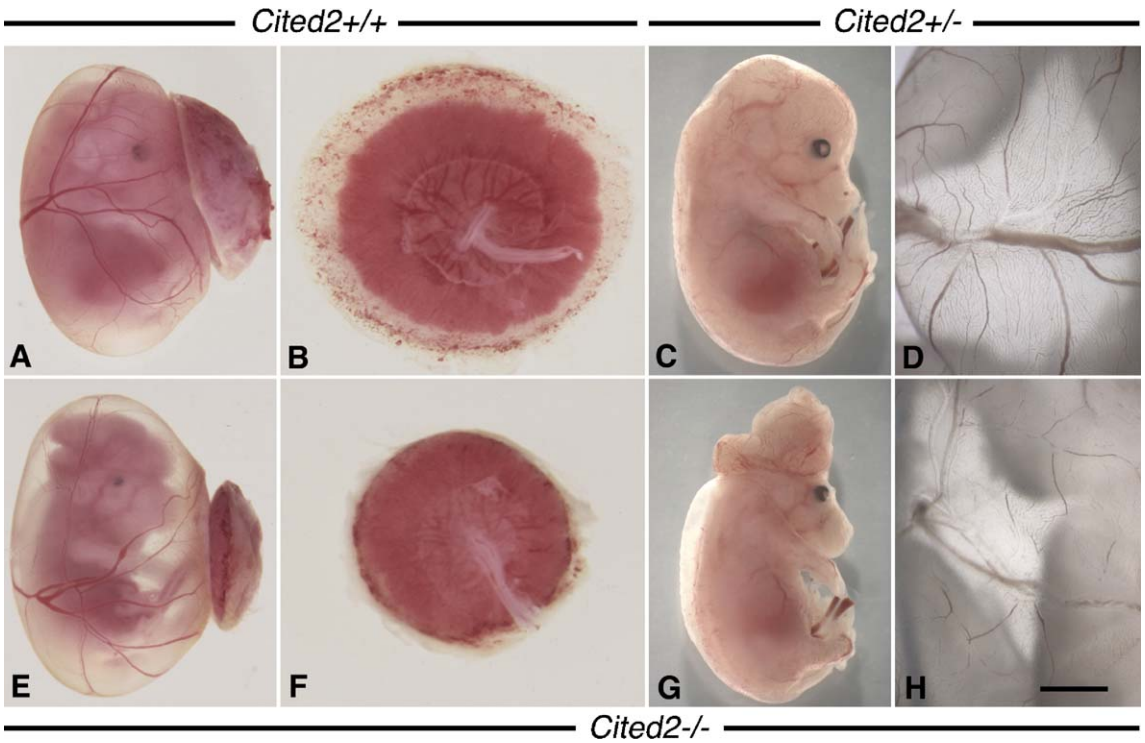

Fig. 1. *Cited2* null conceptuses show several clear phenotypes. Wildtype (A–B), heterozygous (C–D) and *Cited2* null (E–H) conceptuses at 14.5 dpc. Reduced placenta size (F), oedema (G), exencephaly (G) and reduced blood in the yolk sac (H) are evident in the null conceptus compared to control littermates. Scale bar: 290 μm (A and E), 190 μm (B and F), 230 μm (C and G) and 160 μm (D and H).

existence of abnormalities deeper within the developing placenta structure of *Cited2* nulls. Weight measurements of conceptuses at the time of dissection, quantified the size differences between the genotypes (Fig. 2). Placenta weight was significantly reduced ($P < 0.005$–0.0001) in all nulls from 12.5 dpc by up to 38%, and embryo weight from 14.5 dpc by up to 22% ($P < 0.05$–0.001). Reduced embryo weight was observed 2 days after the reduction in placental weight. This suggests that embryo development is compromised due to poor placental formation and function.

Cited2 is required for normal placental morphology

The mouse placenta by 10.5 dpc consists of three layers; proximal to the fetus is the labyrinthine layer, which consists of fetal blood vessels that express *Mest* (Mayer et al., 2000) and maternal blood spaces that are lined by syncytiotrophoblasts and cytotrophoblasts. Distal to this, the spongiotrophoblast layer consists of spongiotrophoblasts and their derivatives, glycogen cells; both these cells type express *4311* (Adamson et al., 2002; Lescisin et al., 1988). Next, there is a layer of secondary TGC, usually large in size, as they are polyploid (Zybina and Zybina, 1996). Various angiogenic, vasodilatory and anticoagulation factors, including placental lactogens (*mPL1, mPL2*), *proliferin* (*PLF*), and *proliferin related protein (Plfr/Prp)*, are expressed by particular subsets of TGC (Carney et al., 1993; Colosi et al., 1987; Hemberger et al., 2003a,b; Linzer et al., 1985). Trophoblasts migrate into locations within the maternal decidua; those expressing the TGC marker *PLF* are present around and within the lumen of the maternal vessels (peri/endovascular trophoblasts), whilst glycogen cells that express *4311*, do not preferentially localise to vessels and thus are located interstitially (Adamson et al., 2002). The peri/endovascular trophoblasts replace the endothelial cells of the maternal vessels; trophoblast cells therefore directly line maternal blood spaces in the placenta.

To analyse the placenta phenotype found in *Cited2* nulls in more detail, we performed RNA in situ hybridisation using marker genes (*4311, mPL2, PLF, Mest*) at 14.5 dpc, on consecutive sections from *Cited2* null ($n = 5$) and wildtype placentas ($n = 3$). We found that each of these genes was expressed in null placentas, indicating that differentiated cell types and the characteristic layers of the mature placenta were formed (Fig. 3). However, there were fewer spongiotrophoblasts, glycogen cells and secondary TGC in *Cited2* null placentas, and the presence of trophoblasts in the decidua was greatly reduced (Figs. 3A–C, E–G, I–K, M–O). Interestingly, we find *mPL2*-expressing cells in the decidua and believe that this is the first time that this expression pattern has been reported. In addition, *Mest* expression indicated that the fetal blood vessels were less densely organised in what appeared to be an expanded labyrinthine layer (Figs. 3D, H, L, P). In order to quantify these observations, the area of specific regions of the placenta was determined: whole placenta, labyrinthine layer, spongiotrophoblast layer, glycogen cells in the decidua, and the decidua (Table 1). Boundaries of the different layers were clearly visualised using the marker *4311*. These measurements confirmed our initial observations and showed that the proportional areas occupied by spongiotrophoblasts and invasive glycogen cells, were greatly reduced (63%

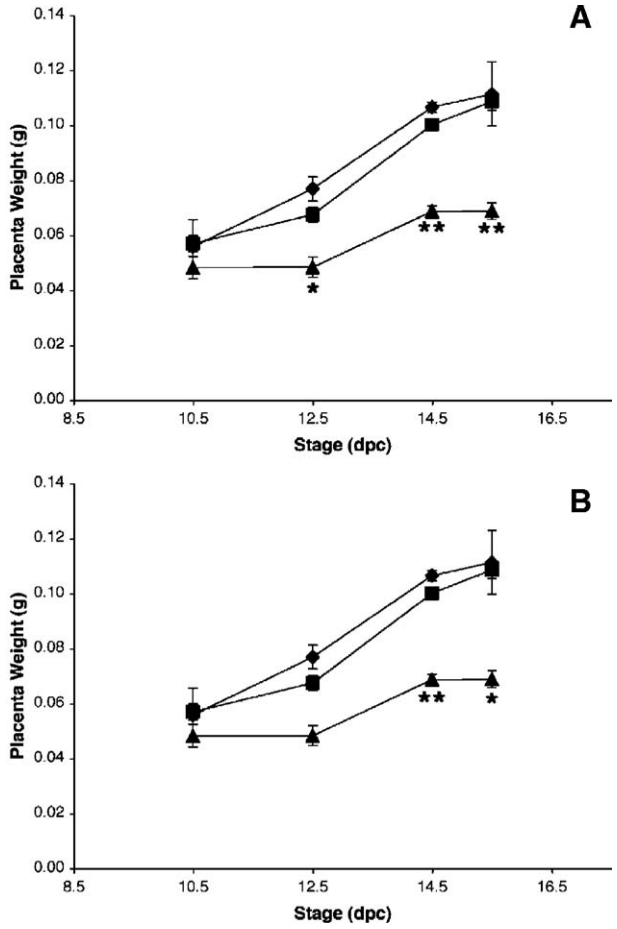

Fig. 2. Placenta and embryo weights are reduced in *Cited2* conceptuses. *Cited2* heterozygous mice were intercrossed and the resulting placentas (A) and embryos (B) weighed upon collection at various stages of gestation. Graphs show the mean weight (and standard error of the mean) for the three genotypes: *Cited2* wildtype (diamonds), heterozygous (squares) and null (triangles). One-way analysis of variance was used to identify differences between genotypes, (*$P \leq 0.005$; **$P \leq 0.0001$).

and 40% respectively, $P < 0.0001$) in *Cited2* nulls, with the labyrinthine layer occupying a greater (9%, $P < 0.05$) proportional area than normal.

Cell proliferation, apoptosis, and levels of p53 are unaffected in Cited2 null placentas

A reduction in trophoblasts in *Cited2* null placentas at 14.5 dpc may occur as a result of reduced cell proliferation and/or increased cell death. Therefore, BrdU incorporation was examined as a measure of DNA synthesis, and active (cleaved) caspase 3 protein expression was assessed to evaluate the extent of apoptosis. The level of p53 protein was also examined since its accumulation in cells inhibits proliferation and/or induces apoptosis (Harris and Levine, 2005). At 11.5 and 12.5 dpc, BrdU was incorporated to different extents into TGC, spongiotrophoblasts, glycogen cells, and trophoblasts and endothelial cells of the labyrinthine layer. At 14.5 dpc, BrdU was incorporated into all these cell types except for secondary TGC, as previously described (Lotz et al., 2004). Active caspase 3 protein was examined at 12.5 dpc, and was only detected in a proportion of decidual cells. p53 protein was detected at 12.5 dpc in secondary TGC, spongiotrophoblasts, glycogen cells, and trophoblasts and endothelial cells of the labyrinthine layer, as previously described (Ren et al., 2005). The proportion of cells expressing these proteins in particular placental cell types was determined. There was no correlation between genotype and the detection of BrdU, active caspase 3, or p53 (data not shown).

Cited2 is expressed in extraembryonic tissues throughout gestation, and is found in both mesoderm-derived and trophoblast cells of the placenta

We have previously shown, by RNA in situ hybridisation, that *Cited2* is expressed in extraembryonic endoderm and mesoderm, and particularly in the blood islands of the visceral yolk sac (Dunwoodie et al., 1998). We extended this expression analysis, taking advantage of the fact that expression of the reporter gene *lacZ* is under control of the endogenous *Cited2* promoter in the *Cited2-lacZ* mice. *Cited2-lacZ* expression was compared between *Cited2* heterozygous (one copy of *lacZ*), and null (two copies of *lacZ*) individuals. *Cited2-lacZ* expression varied in level but not the distribution between the two genotypes, thus *Cited2-lacZ* expression in *Cited2* nulls is shown (Figs. 4 and 5). *Cited2-lacZ* expression was observed at 7.0 dpc in a central core of the future placental tissue, the EPC (Figs. 4A, B). Although low levels of *Cited2-lacZ* expression were seen throughout most of the embryo and extraembryonic tissues, stronger staining was clear in the posterior nascent mesoderm (Figs. 4A, B) and in the developing blood islands (Fig. 4B). By the 9-somite stage (8.5 dpc), two domains of *Cited2-lacZ* staining were visible in the forming placental region (Fig. 4C). These domains of expression were in two distinct cell types. The upper expression domain was in trophoblast cells of the EPC. The lower expression domain was in mesoderm cells of the allantois, and interestingly *Cited2-lacZ* expression was particularly strong in the allantoic mesoderm in contact with the trophoblasts of the chorion (see also Dunwoodie et al., 1998). No staining was apparent at all in the trophoblasts of the chorion, which lay between the EPC and allantoic mesoderm (Fig. 4C). A continuation of this expression pattern was observed at 9.5 dpc (Fig. 4E), and in addition expression was readily detected in TGC (Figs. 4D, F). In the yolk sac, *Cited2-lacZ* expression was observed in the mesodermal layer. Although expression was strongly localised to the vessel walls in flatmount preparations, it was difficult to identify expression in endothelial cells lining the vessels upon sectioning (Figs. 4G, H). At 14.5 dpc, *Cited2-lacZ* expressing cells were found in all trophoblast-derived layers of the mature placenta, but were absent from maternally derived tissue (Fig. 5A). Strong *Cited2-lacZ* expression was observed in the TGC layer, and in cells directly lining the maternal blood spaces as they traversed the spongiotrophoblast and labyrinthine layers (Figs. 5B, E). This aspect of the expression pattern closely resembled that of *PLF* (Fig. 3C). *Cited2-lacZ* expression was also particularly strong in spongiotrophoblast cells (Figs. 5A, B). In addition, *Cited2-lacZ* was expressed in glycogen cells, and expression was maintained as they migrated into the decidua

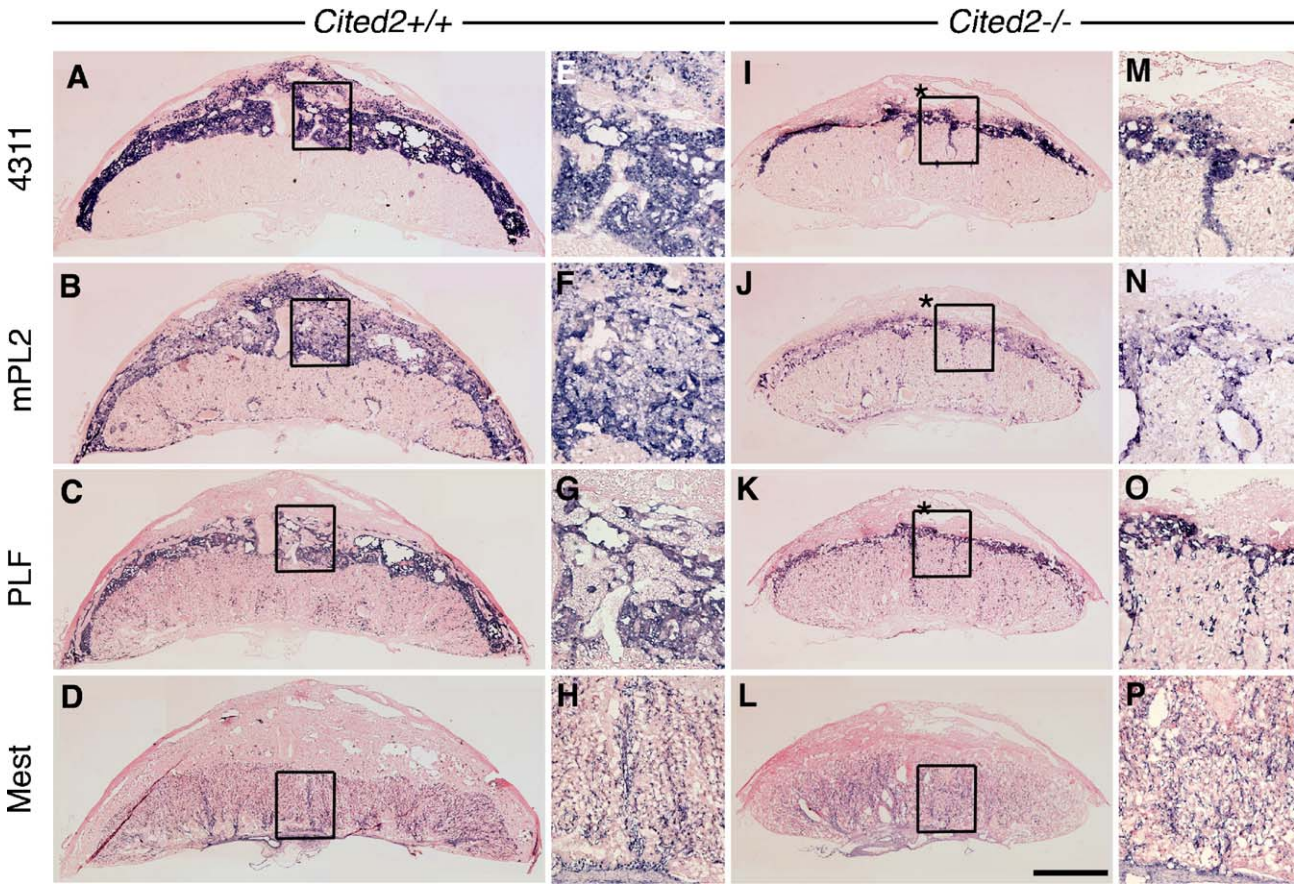

Fig. 3. Spongiotrophoblast and TGC layers in particular are reduced in *Cited2* nulls at 14.5 dpc. Transverse sections of *Cited2* wildtype (A–H) and null (I–P) placentas. *4311* marks spongiotrophoblasts and glycogen cells. *mPL2* and *PLF* label subsets of secondary TGC. The greatly reduced number of invasive glycogen cells, *mPL2*- and *PLF*-expressing cells in decidua of null placentas is marked with an asterisk. *Mest* marks the endothelial cells of the fetal blood vessels in the labyrinthine layer. Small panels to the right of each placental section are magnifications of the boxed areas. Scale bar: 130 μm (A–D and I–L) and 410 μm (E–H and M–P).

(Figs. 5C, D). In this respect, *Cited2* expression closely correlated with patterns of *4311* and *mPL2* expression (Figs. 3A, B). In the labyrinth, *Cited2-lacZ* was expressed in trophoblast cells, and in the endothelial and smooth muscle cells of the fetal vessels (Figs. 5B, E). However, expression was strikingly absent from the columnar epithelium at the base of the labyrinthine layer, the chorionic plate (Figs. 5F, G). Patterns of staining for *Cited2-lacZ* in the fetal vessels (allantois-derived) and for alkaline phosphatase in the chorionic trophoblasts were mutually exclusive in this region (compare Figs. 5G and H). The *Cited2-lacZ* expression presented in Fig. 5 was generated from the *Cited2* null conceptuses as two copies of *lacZ* made detection of expression more robust than one copy present in *Cited2* heterozygotes. The *Cited2-lacZ* expression domains were the same in *Cited2* heterozygous and null placentas, however as previously described there are fewer TGC, spongiotrophoblasts and trophoblast derivatives in the decidua.

In summary, *Cited2-lacZ* was expressed in placental tissue throughout gestation. *Cited2-lacZ* staining was seen in most differentiated trophoblast cell types; with the regions of strongest expression correlating with the layers most affected in the *Cited2* nulls. Staining was also seen in the extraembryonic mesoderm that forms the fetal vessels. It is relatively unusual for a gene to be expressed in both mesoderm-derived and trophoblast compartments of the placenta (Simmons and Cross, 2005). Interestingly, *Cited2-lacZ* was not expressed in the chorionic trophoblasts that interact with the allantoic mesoderm. The chorion layer undergoes buckling and invagination to allow the fetal vessels to penetrate the labyrinth, and also contains TS cells.

Cited2 is expressed in differentiating trophoblast cells in culture

TS cells can be grown in culture, and maintained in a proliferative state if given the appropriate growth factors (Guzman-Ayala et al., 2004; Tanaka et al., 1998). TS cells can also be induced to differentiate into various trophoblast cell types, and express markers characteristic of these cell types. Since *Cited2* was strongly expressed in the placenta, we addressed whether it was also expressed in cultured trophoblast cells (Fig. 6). In particular, we examined whether *Cited2* was expressed in proliferating TS cells, or in differentiating trophoblasts. We found that *Cited2* was strongly expressed in differentiating trophoblast cells, in a pattern similar to *4311* and *mPL1/2* expression (Fig. 6). However, expression of *Cited2* in the colonies of small, tightly packed TS cells was negligible (Fig. 6A). Therefore, we conclude that *Cited2* is expressed in TGC and spongiotrophoblasts, but largely absent from TS cells, both in vivo and in vitro.

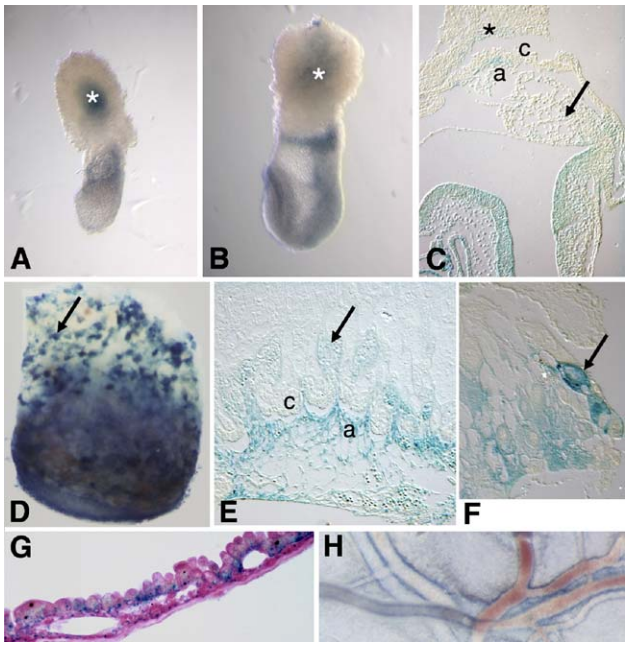

Fig. 4. *Cited2-lacZ* is expressed in placental tissues from early stages. Lateral views and sagittal section of embryos, anterior to the left (A–C). (A) Full length streak stage. (B) Late headfold stage. (C) 9-somite stage (8.5 dpc), allantois (arrow). (D) Inner lining of the decidua at 9.5 dpc, TGC (arrow). (E, F) Sagittal sections of 9.5 dpc placenta, EPC-derived trophoblasts (arrow in panel E), TGC (arrow in F). (G) Flatmount view of a yolk sac from a 14.5 dpc. H: Transverse section of 15.5 dpc yolk sac. *Cited2* null (A–F,H), *Cited2* heterozygous (G). EPC (*), chorion (c), allantoic mesoderm (a). Scale bar: 275 μm (A), 375 μm (B), 170 μm (C), 1.1 mm (D), 400 μm (E, F), 180 μm (G, H).

Loss of Cited2 affects early interaction of the chorion and allantois

The labyrinth is named because of the intricate structure of convoluted and interconnecting maternal and fetal blood spaces within this part of the placenta. Formation of the labyrinth is initiated by interaction of two formerly spatially separate tissues: the allantois and the chorion. The allantois is extra-embryonic mesoderm that extends from the posterior of the embryo to contact the trophoblast cells of the chorionic plate (see Gardner, 1983). Interaction of the chorion and allantois causes buckling and invagination of the chorion; this enables the fetal endothelial cells, and later blood vessels, to penetrate and form the labyrinth (see Downs, 2002; Cross et al., 2003). The endothelial and smooth muscle cells of the fetal vessels in the labyrinth are therefore derived from allantoic mesoderm.

At 10.5 dpc the labyrinth was proportionally smaller in *Cited2* nulls (data not shown), suggesting early defects in formation of this layer. *Cited2-lacZ* was expressed particularly strongly in the allantoic mesoderm cells that interact with the chorion at 8.5 dpc (Fig. 4C), and expression was maintained in the walls of fetal vessels throughout the labyrinth (Figs. 5B, F, G). We therefore examined whether any defects in the interaction between the chorion and allantois were evident in *Cited2* null placentas. Sections showed that buckling of the chorionic plate at 9.5 dpc was noticeably reduced in *Cited2* nulls (Figs. 7A, B). Concomitantly, penetration of fetal vessels into the early placenta was reduced, with the vessels remaining at the base of the placenta. These results therefore demonstrate that interaction of the chorion and allantois is compromised in the absence of *Cited2*. However, since placentas of *Cited2* nulls continued to develop, and the labyrinth expanded to occupy a large fraction of the 14.5 dpc placenta (Table 1), it appears that this early defect represents a delay in vessel penetration that was overcome.

Since *Cited2-lacZ* was expressed in the walls of the fetal vessels throughout the labyrinth, we examined whether the integrity of these vessels was compromised in nulls. Such defects could account for the reduced ability of the vessels to penetrate through their environment. In wildtype placentas at 11.5 dpc, smooth muscle actin (SMA) strongly stained multiple densely packed layers of the large fetal vessels at the base of the labyrinth (Fig. 7D). SMA expression followed the fetal vessels as they branched and penetrated into the labyrinth, right up to the border with the spongiotrophoblast layer (Fig. 7E). Expression associated with smaller fetal vessels was also seen scattered fairly evenly throughout the labyrinth (Fig. 7F). No staining was seen associated with maternal blood spaces in the labyrinth, consistent with the fact that maternal blood directly contacts trophoblast cells, with no intervening endothelial or smooth muscle cells. In *Cited2* null placentas, the density of cells that express SMA in the wall of the large fetal vessels was clearly reduced, and these cells were less well organised (Fig. 7G). The density of SMA positive cells was also greatly reduced in the walls of the vessels as they traversed the labyrinth (Fig. 7H). Finally, the overall number of scattered clumps of SMA staining was reduced throughout the labyrinth, indicating reduced density of fetal vessels (Fig. 7I). Nevertheless, staining was observed in all regions of the labyrinth, signifying that the fetal vessels were still able to traverse the entire layer, and reach close to the spongiotrophoblast border.

Cited2 is required for normal organisation of the fetal capillaries in the labyrinth

Since the labyrinth is the site of gaseous and nutrient exchange between the mother and fetus, the arrangement of fetal blood vessels and maternal blood sinusoids within the labyrinth is of fundamental importance. Altered SMA localisation (Fig. 7), together with initial observations at the time of dissection, suggested that fetal vasculature was abnormal in *Cited2* null placentas. We extended this analysis, and gained a much clearer understanding of the fetal and maternal circulations in three dimensions by generating vascular casts using resin infiltration. Casts of the fetal vasculature were generated by injection of resin into the umbilical artery (blue) and vein (red) at 14.5 dpc (Fig. 8). As previously described (Adamson et al., 2002; Rodriguez et al., 2004), these casts showed that in wildtype placentas the arterioles branched and traversed through the entire labyrinth to the spongiotrophoblast layer, before forming a dense mass of capillaries that extended back towards the base of the placenta. The venuole branches, however, only penetrated a short distance into the labyrinth and immediately formed a mass of capillaries that extended up to meet those derived from the arterioles (Figs. 8A, B). The fetal vasculature of many *Cited2* null placentas was so impaired that resin injection was not

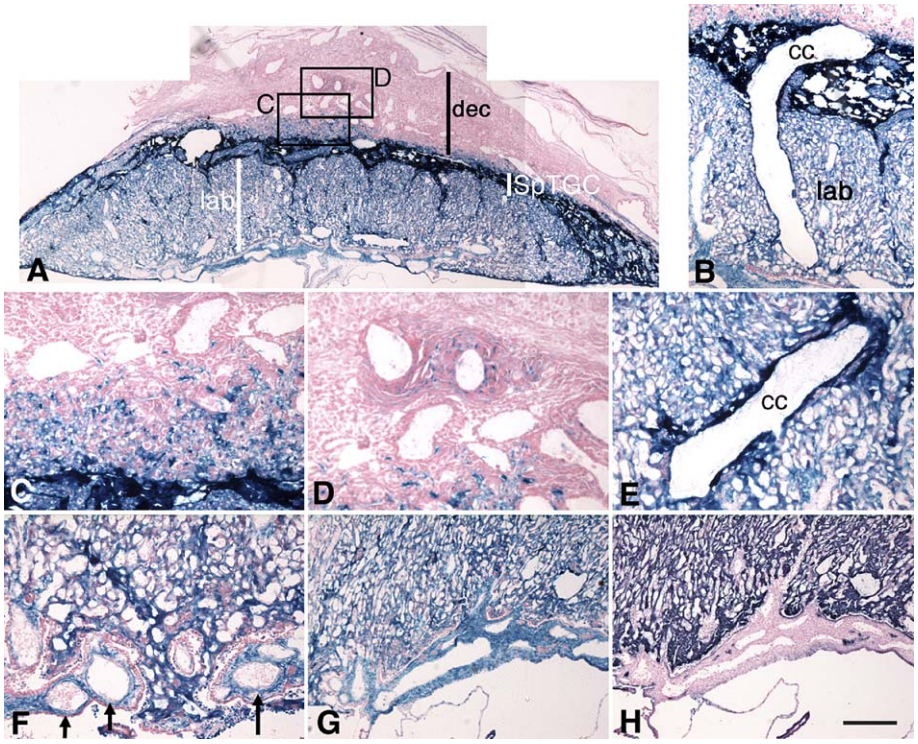

Fig. 5. *Cited2-lacZ* is expressed in both mesoderm-derived and trophoblast cells of the placenta. Transverse cryosections of 14.5 dpc *Cited2* null placentas, (maternal decidua to the top) counterstained with fast red (A–F are of same specimen and G–H are of a littermate). (A) Whole placenta. (B) Maternal central canal (cc), traversing the TGC, spongiotrophoblast and labyrinthine layers. (C) Proximal decidua (boxed in panel A), showing interstitial migration of glycogen cells. (D) Adjacent view to panel C (boxed in panel A), showing more distal decidua. (E) Magnified view of a maternal central canal (cc), lined by trophoblast cells, in the labyrinth. (F) Large fetal vessels (arrows) at the base of the labyrinth. (G, H) Consecutive sections at the base of the labyrinth showing *Cited2-lacZ* (G) and alkaline phosphatase expressing trophoblasts (H). Maternal decidua (dec), spongiotrophoblast and TGC layer (SpTGC), labyrinthine layer (lab). Scale bar: 750 μm (A), 375 μm (B, G, H) and 190 μm (C–F).

possible. Nevertheless, good resin infiltration was achieved in 20 of the less affected *Cited2* null placentas. In these casts, the branching pattern and size of the large arterioles and venuoles appeared relatively unaffected (Figs. 8C, E). However, the overall arrangement of the vessels was generally disorganised, as seen when the casts were cut in half and viewed side on (Figs. 8D, F). Notice also that in one of the *Cited2* null placentas shown, the umbilical vein was bifurcated, with two vessels leaving the placenta (Fig. 8D). The most dramatic difference we found was in the capillaries formed at the tips of the venuoles and arterioles. There was variability in the severity of this phenotype, but in 12/20 casts, the capillaries were severely stunted, disorganised and irregular in shape and thickness. Capillaries with very different diameters could be seen emerging from the same vessel. Furthermore, capillary distribution was not uniform within each null placenta, with some parts of the labyrinth more affected than others. In the most severe cases, almost no capillaries formed at all. This loss of capillaries was clearly evident by viewing with light microscopy at low magnification, but was vividly illustrated using scanning electron microscopy (compare Figs. 8G, H with I–L).

Maternal vascular casts were generated following resin infusion from the arterial ($n = 30$; Fig. 9) or venous ($n = 8$; data not shown) side. A lateral view of the vascular cast filled from the arterial side shows the arrangement of maternal arteries and trophoblast-lined blood spaces of the placenta (Figs. 9A, B). The organisation of the radial arteries, spiral arteries and central canals was similar to that previously described (Adamson et al., 2002; Rodriguez et al., 2004) and appeared to be the same regardless of the genotype. In wildtype placental casts viewed from the base, the canal branches and anastomosing sinuses radiated out to form an even circular disc. However, in 5/8 casts from null placentas and 3/11 casts from *Cited2* heterozygous placentas, the overall diameter of the disc was slightly smaller, with the rim disrupted and uneven (data not shown). The sinusoids in placentas of all genotypes were well branched and fairly regular in size ($n = 30$). However, the sinusoids were slightly larger in 5/8 casts from null placentas and 2/11 casts from *Cited2* heterozygous placentas, compared to their littermates (Figs. 9C, F). Thus, casts generated from *Cited2* null placentas showed more subtle defects in the maternal blood spaces compared to the fetal vasculature.

The distribution of cells low in oxygen is not altered in Cited2 null placentas

Expression of *Cited2* is induced under conditions of low cellular oxygen directly through HIF1α activity (Bhattacharya et al., 1999). We therefore examined whether *Cited2* expression correlated with regions of low oxygen in the placenta. Little is known about oxygen levels in mouse placental cell types in vivo. We have identified that TGC, spongiotrophoblasts, and glycogen cells in the decidua have low levels of oxygen (≤2%; Fig. 10). Cells in the labyrinthine layer, and those that surround the

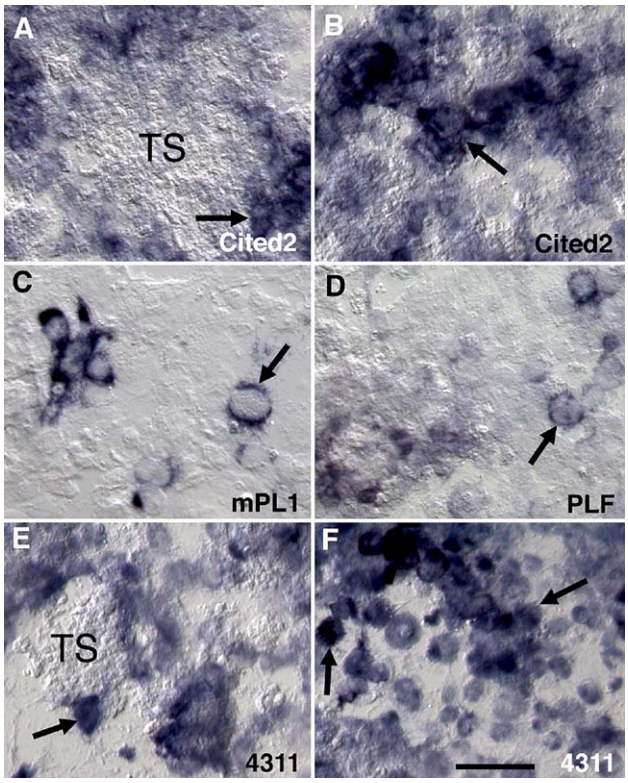

Fig. 6. *Cited2* expression in cultured trophoblast cells correlates with the in vivo expression pattern. Light micrographs showing clusters of wildtype trophoblast cells in culture. Cells have been fixed and processed for in situ hybridisation with *Cited2* (A, B), *mPL1* (C), *PLF* (D) and *4311* (E, F). TS cells are present as tight colonies of small cells (best examples are in panels A, E; TS) whereas differentiated trophoblast are large and rounded (present in all panels; arrows). Scale bar: 75 μm.

maternal blood vessels in the decidua, contained >2% oxygen. In *Cited2* null placentas, we observed a reduction in the number of cells that contain ≤2% oxygen. However, this is likely to be due to the fact that the cells that contain low levels of oxygen (≤2%; TGC, spongiotrophoblasts and glycogen cells) are those that are reduced in *Cited2* null placentas. There were no other discernable differences in the distribution of low oxygen containing cells between genotypes at 9.5 (data not shown) and 14.5 dpc.

Cited1 genotype does not influence the placental phenotype of Cited2 null mice

We have previously reported that mice deficient for another member of the *Cited* gene family, *Cited1*, also die of placental defects (Rodriguez et al., 2004). *Cited1* is expressed in TGC, spongiotrophoblasts, and a subset of labyrinthine trophoblasts. *Cited1* null mice die immediately after birth and show intrauterine growth restriction. Resin casts generated of *Cited1* null placentas at 16.5 dpc showed very little difference in fetal vasculature but grossly enlarged maternal sinusoids (Rodriguez et al., 2004). Given that both genes are expressed in trophoblasts, it is possible that *Cited1* function in trophoblasts may be able to partially compensate for the lack of *Cited2*. To test this hypothesis, we generated mice lacking both *Cited1* and *Cited2*. Mice were intercrossed to produce a range of genotypes, and embryos and placentas were analysed at 14.5 dpc (Table 2). The number of *Cited2* null embryos recovered was significantly less than expected according to Mendelian ratios, and embryo loss at this stage did not correlate at all with *Cited1* genotype (data not shown). In each case, embryo and placenta weight was less in *Cited2−/−* compared to *Cited2+/+* except in the case of *Cited1−/−*. In males null for *Cited1 (Cited1−/Y)* there was no impact on the *Cited2* phenotype when compared with *Cited1* wildtype males *(Cited1+/Y)*. Likewise in females null for *Cited1 (Cited1−/−)* there was no impact on the *Cited2* phenotype when compared with *Cited1* wildtype females *(Cited1+/+)*. Taken together, these findings show that loss of *Cited1* had no obvious effect on the severity of phenotype observed in *Cited2* nulls, and suggest that these two genes act independently.

Discussion

Here, we describe a novel role for *Cited2* in the development of the placenta. In the absence of *Cited2* in the mouse, placentas are small, have fewer TGC, spongiotrophoblasts, glycogen cells and *PLF*-expressing cells in the decidua, and reduced fetal vascular development. *Cited2* is expressed in fetal endothelial cells, TGC, spongiotrophoblasts, invasive glycogen cells, but not in TS cells. We and others have also described a requirement for *Cited2* in cardiovascular development (Bamforth et al., 2001, 2004; Martinez-Barbera et al., 2002; Weninger et al., 2005; Yin et al., 2002). Since *Cited2* is expressed in both the developing heart and placenta, it is likely that many of the defects that occur in these organs are a direct result of the loss of *Cited2* expression. However, placental insufficiency may have an effect on heart development, or indeed abnormal heart development may impact on placental development. Several mouse mutants with defects in the placenta also exhibit an abnormal heart phenotype. For some of these, the embryonic heart defects are secondary to placental failure, since they are prevented by the provision of wildtype trophoblasts in tetraploid chimeras (Adams et al., 2000; Barak et al., 1999; Ihle, 2000). Supply of a wildtype placenta has also been sufficient to rescue defects in other organs, for example many aspects of the *Rb* phenotype, including in the nervous system and haematopoiesis, are rescued in this way (de Bruin et al., 2003; Wu et al., 2003). Tetraploid aggregation experiments (Rossant and Cross, 2001) or conditional removal of *Cited2* from the heart or placenta using Cre-LoxP technology should establish if the loss of *Cited2* in the placenta affects heart development, and vice versa.

Formation of TGC, spongiotrophoblasts and glycogen cells

Cited2 null placentas have fewer TGC, spongiotrophoblasts, and invasive glycogen cells, and *PLF*-expressing cells in the decidua. This could occur if too few progenitor cells differentiate to form these specific trophoblast cell types. TS cells reside within the extraembryonic ectoderm, and later its derivative the chorion (Tanaka et al., 1998; Uy et al., 2002). They provide the ectoplacental cone (EPC) with progenitors that give rise to TGC and spongiotrophoblasts (Carney et al., 1993; Johnson and Rossant, 1981; Rossant et al., 1978). TS cells can be derived from blastocysts or 6.5 dpc

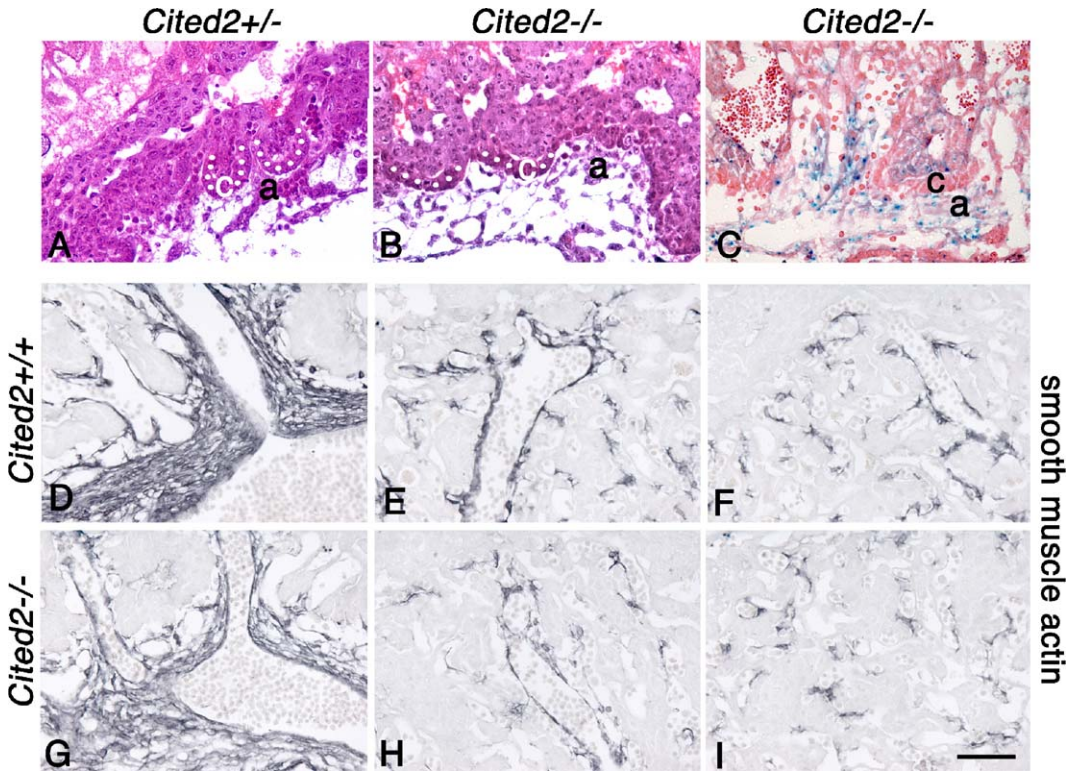

Fig. 7. Early interaction of chorion and allantois is affected in *Cited2* null placentas. (A, B) Haematoxylin and eosin staining of sections from *Cited2* heterozygous (A) and null (B) placentas at 9.5 dpc. Views show interaction of the chorion (c) and allantoic mesoderm (a), at the base of the developing labyrinth. (C) *Cited2-lacZ* expression in a *Cited2* null placentas at 9.5 dpc placenta. (D–I) Immunostaining for smooth muscle actin in *Cited2* wildtype (D–F) and null (G–I) placentas at 11.5 dpc. Views show large fetal vessels at the base of the labyrinth (D, G) or smaller vessels throughout the labyrinth (E, H, F, I). White dots show the degree to which the chorion has buckled. Scale bar: 120 μm (A–C), 660 μm (D–I).

extraembryonic ectoderm, and continue to proliferate in vitro if provided with appropriate growth factors usually provided by the adjacent epiblast (Guzman-Ayala et al., 2004; Tanaka et al., 1998). TS cells in culture can also be induced to differentiate and can form various trophoblast subtypes (Hughes et al., 2004; Maltepe et al., 2005; Tanaka et al., 1998). Since *Cited2* is not expressed in TS cells in vitro, nor in vivo in the extraembryonic ectoderm or chorion, it is more likely that it functions in cells derived from TS cells. Gene expression studies indicate that both TGC and spongiotrophoblasts form from an EPC progenitor that expresses *4311*. However, added complexity appears to exist concerning trophoblast differentiation, as lineage-tracing studies show that not all TGC are derived from this common progenitor (see Watson and Cross, 2005). Mouse mutants often show disproportionate differentiation biased towards TGC or spongiotrophoblasts (Guillemot et al., 1994; Plum et al., 2001; Shi et al., 2004; Tamai et al., 2000). Furthermore, different oxygen levels in cultures of TS cells can promote differentiation into either TGC or spongiotrophoblasts, and thereby regulate the fate of the trophoblast progenitors; this response is lost in TS cells derived from *Hif1*β mutants (Adelman et al., 2000). In contrast, mutations in *Rap250* (Antonson et al., 2003) or *Hsf1* (Xiao et al., 1999) cause a loss in only spongiotrophoblast cells, without affecting the TGC population, suggesting that they act further downstream in the spongiotrophoblast commitment pathway. Finally, *p185/Cul7* mutants cause a decrease in both cell types (Arai et al., 2003) and a hypomorphic allele of *Nodal* causes an increase in both cell types (Ma et al., 2001), suggesting that these two genes may act earlier in the pathway to affect the total number of EPC progenitors formed. *Cited2* null placentas have reduced spongiotrophoblasts, secondary TGC, invasive glycogen cells, and *PLF*-expressing cells in the decidua. This suggests that trophoblast proliferation is reduced and/or that apoptosis is increased. We examined both these features between 11.5 and 14.5 dpc, and found no consistent difference between placentas of different *Cited2* genotype. Therefore it is possible that there is reduced production of TGC and spongiotrophoblasts from their progenitor cells in the EPC.

Since evidence suggests that *Cited2* may function in the EPC to generate spongiotrophoblasts and TGC, we established EPC explant cultures; there were no detectable differences in cell survival or differentiation between *Cited2* genotypes. It is possible that an EPC phenotype might be detected in the absence of *Cited2* only when the explants are cultured under physiologically relevant low oxygen conditions, since *Cited2* is associated with hypoxic signalling.

Functional consequences of reduced trophoblasts in Cited2 null placentas

The female physiology undergoes major adaptive changes during gestation; the TGC are central to this through the production of luteotrophic, lactogenic, vasoactive, and anticoagulative factors (Cross et al., 2002; Linzer and Fisher, 1999). TGC

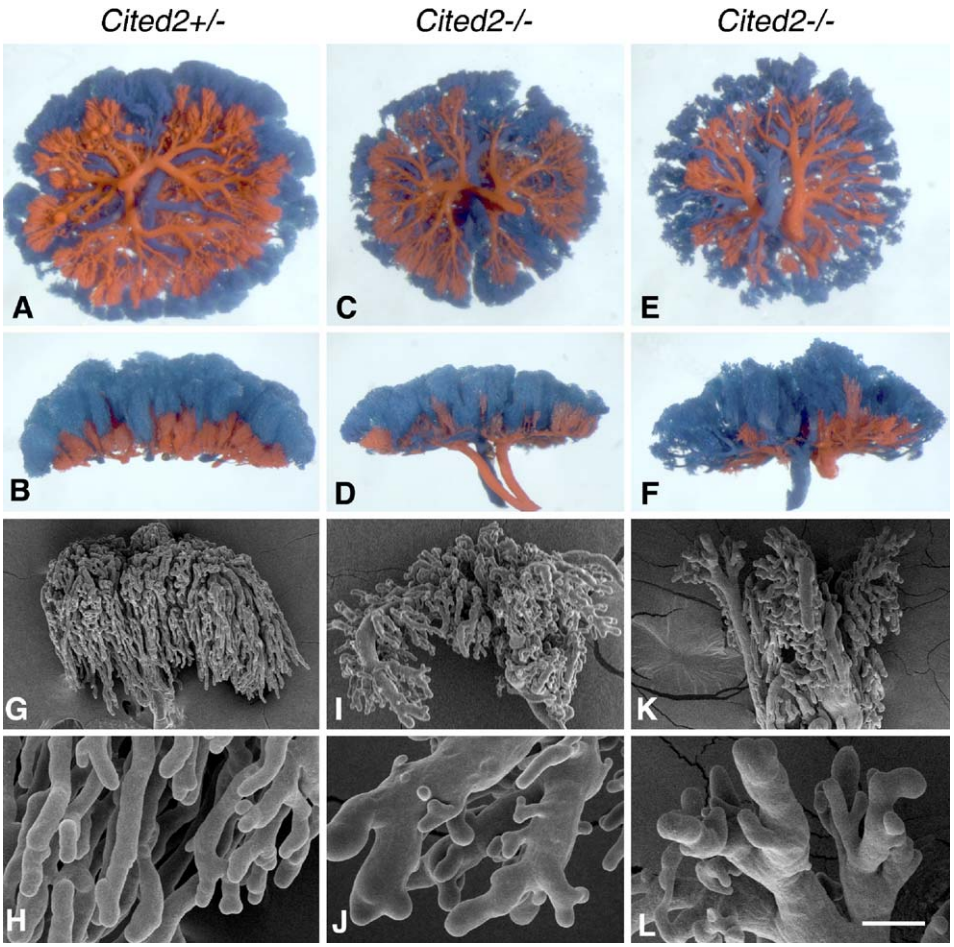

Fig. 8. Fetal capillary formation is severely affected in the labyrinth of *Cited2* null placentas. (A–F) Light micrographs of resin casts generated from *Cited2* heterozygous (A, B) and null (C–F) placentas viewed from the base (A, C, E) and side (B, D, F). Fetal vasculature in the labyrinth was filled by injection into the umbilical artery (blue) and vein (red) at 14.5 dpc. (G–L) Scanning electron micrographs of the same casts, showing the capillaries that form at the tips of the arterioles. Scale bar: 1.4 mm (A, C, E), 1.3 mm (B, D, F), 290 μm (G, I), 50 μm (H, J, L) and 200 μm (K).

also differentiate into invasive endovascular trophoblasts that displace the endothelial cells of the spiral arteries that supply the placenta with maternal blood (Adamson et al., 2002; Hemberger et al., 2003a). Given that *Cited2* null placentas exhibit a reduction in both TGC and *PLF*-expressing endovascular invasive TGC, one might have predicted that a consequence of this might be disruption of the maternal blood spaces. This was not the case; a modest increase in the size of maternal sinusoids in the labyrinthine layer was observed. This reduction in surface area would lead to less efficient exchange between the fetal and maternal blood; however, the observed expansion of the labyrinthine layer may have gone someway towards ameliorating this.

The function of the spongiotrophoblast layer is poorly understood. However, its compact structure suggests that it could act as support for the developing villous structures of the labyrinth (Cross et al., 2002; Iwatsuki et al., 2000). In addition, cross-talk clearly exists between the spongiotrophoblast and cells of the labyrinthine layers. *Ipl* and *Esx1* are expressed only in the labyrinth layer and not the spongiotrophoblasts, but their deletion results in an expanded spongiotrophoblast layer (Frank et al., 2002; Li, 1998). Spongiotrophoblasts also express anti-angiogenic factors such as soluble Flt1 (He et al., 1999) and proliferin-related protein (Prp; Jackson et al., 1994; Linzer and Nathans, 1985), which are specific antagonists of VEGF and proliferin, respectively.

Glycogen cells are derived from the spongiotrophoblast layer. They migrate into the decidua after 12.5 dpc but remain interstitial, and can sometimes travel much further distances than endovascular TGC invasion (Adamson et al., 2002). Glycogen cells become the main source of IGF-II within the placenta in the second half of gestation (Redline et al., 1993) and actually require IGF-II for their differentiation (Lopez et al., 1996). *Igf2* mutant embryos show intrauterine growth restriction, as well as reduced placental size (DeChiara et al., 1991; Lopez et al., 1996). Importantly, deletion of just the placenta-specific transcript of *Igf2* (P0) also causes fetal growth restriction, illustrating the importance of glucose transport and glycogen metabolism by the placenta for sustaining growth of the embryo during later stages of gestation (Sibley et al., 2004). It has also been proposed that glycogen cells have a protective role, allowing maternally and fetally derived cells to co-exist in the placenta (Zybina, 1988). Numerous mutants show a disruption in glycogen cell number,

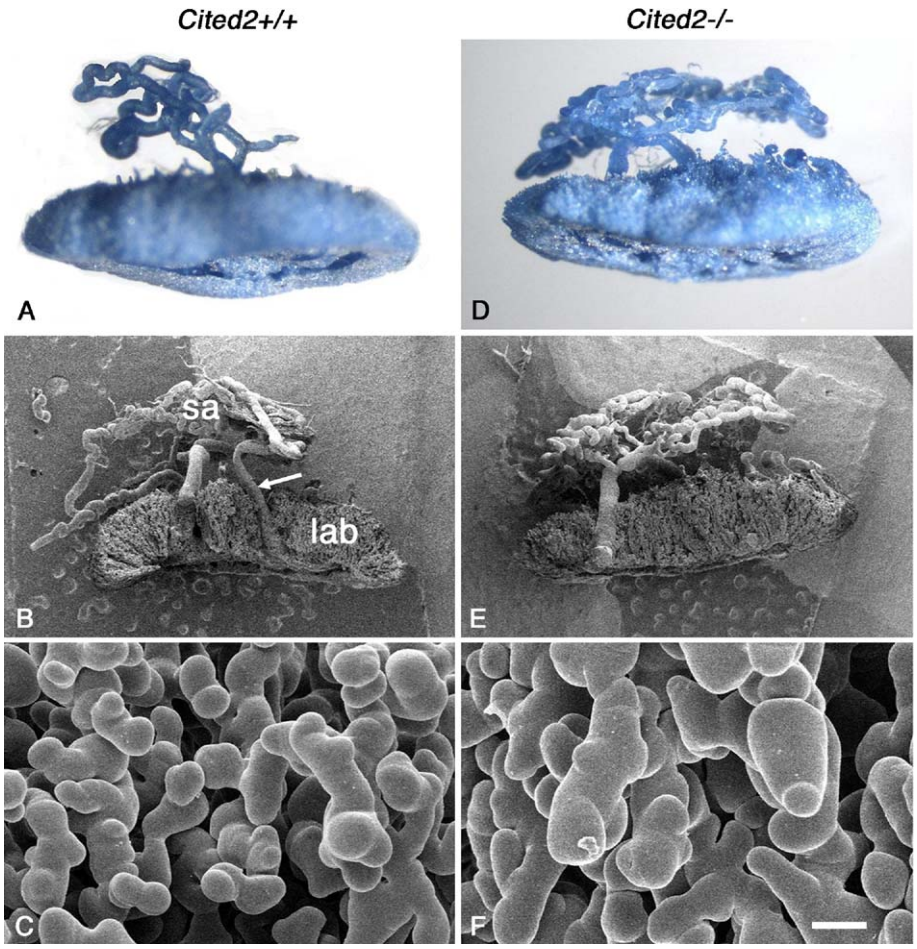

Fig. 9. *Cited2* null placentas show no major defects in maternal blood spaces. Resin casts of maternal blood spaces generated from *Cited2* wildtype (A–C) and null (D–F) placentas, filled from the arterial side at 14.5 dpc. Light micrographs (A, D) and scanning electron micrographs (B, E) show lateral views of the whole casts. Scanning electron micrographs at high power show the sinusoids (C, F). Maternal spiral arteries (sa), central canal (arrow), labyrinthine layer (lab). Scale bar: 1 mm (A, D), 1.25 mm (B, E) and 25 μm (C, F).

but this is normally coupled with a similar defect in the spongiotrophoblast layer itself (Frank et al., 2002; Li and Behringer, 1998; Yang et al., 2003). Effects on both spongiotrophoblasts and TGC number are also evident in cloned mouse conceptuses (Tanaka et al., 2001). However, *Igf2* nulls show a loss in glycogen cells without prior reduction in spongiotrophoblasts, whereas *p57kip2* mutants have an increased spongiotrophoblast layer but no change in glycogen or TGC numbers (Takahashi et al., 2000). Since the number of spongiotrophoblast cells formed in *Cited2* nulls is clearly reduced, it is no surprise that the number of glycogen cells is also reduced. However, *Cited2* may also affect differentiation of the glycogen cells themselves, since any glycogen cells that did form, remained close to the spongiotrophoblast layer and were apparently unable to migrate into the maternal decidua.

Cited2 and the labyrinth

Co-ordinated infiltration of maternal and fetal blood circulations into the trophoblast scaffold in the labyrinth ensures maximal exchange of oxygen, nutrients and waste between the mother and fetus. The labyrinthine layer contains numerous trophoblast cell types, many of which can be identified based on their morphology and location with respect to maternal and fetal blood spaces (Cross, 2000). Cells lining the maternal blood spaces are mononuclear and *mPL2* positive. Adjacent to this are two layers of syncytiotrophoblasts, and then a layer of fetal endothelial cells (Cross, 2000).

Initial formation of the labyrinthine layer is an active process, requiring participation of both chorionic trophoblast and allantoic mesoderm. Following interaction of these two initially spatially separate tissues, the chorion folds and buckles to form villi, creating space into which the fetal blood vessels grow from the allantois (Cross et al., 2003; Watson and Cross, 2005). *Cited2* is strongly expressed in the allantoic mesoderm cells that directly contact the chorion, but not in the chorion itself. *Cited2* mutant placentas show defects in these early interaction events, since the chorion is relatively flat and unfolded. However, these early defects are apparently overcome, and the labyrinth layer continues to develop into a more elaborate structure even in the absence of *Cited2*. The size, number and arrangement of blood spaces in the mature labyrinth are a consequence of the extent of trophoblast branching (Watson and Cross, 2005). Phenotypes described as vascular defects may therefore often be secondary

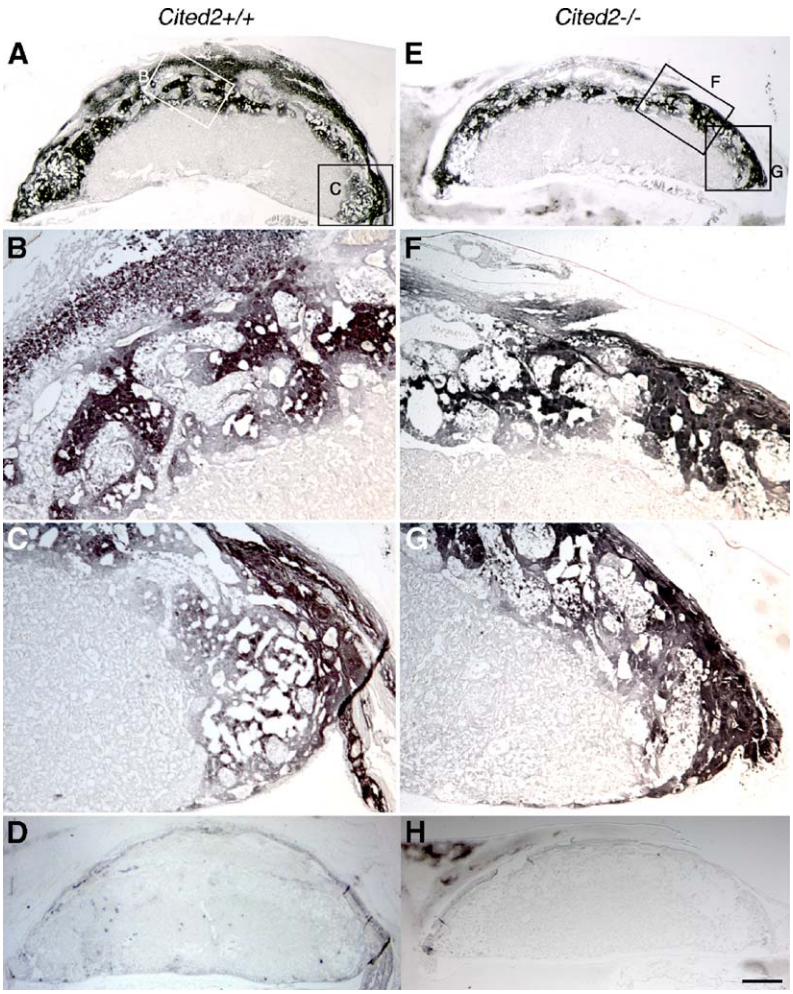

Fig. 10. A comparison of cells containing low levels of oxygen in *Cited2* wildetype and null placentas. Transverse sections of *Cited2* wildtype (A–C) and null (E–G) placentas at 14.5 dpc stained for cells with low (≤2%) oxygen content. (D, H) represent secondary antibody only controls of sections adjacent to panels A and E respectively. Boxed areas in panels A and E magnified in panels B, C , F and G. Scale bar: 1 mm (A, D), 180 μm (B, C, E, F).

consequences of a failure in trophoblast branching or an over proliferation of labyrinth trophoblast cells (Wu et al., 2003). The expression pattern of *Cited2* is relatively unusual in that it is expressed in both trophoblast cells and in mesoderm-derived cells of the fetal vessels that penetrate the placenta. The defects in fetal vessel integrity and in formation of fetal capillaries observed in *Cited2* null placentas might therefore be a primary effect of loss of *Cited2* expression. We will be able to directly address this using a conditional null allele of *Cited2* that we have generated but not yet characterised.

Does the Cited2 null phenotype relate to its putative role in hypoxic signalling?

Many cellular events that lead to development and remodelling of the growing placenta are triggered by oxygen. Oxygen content is a key signal, capable of influencing differentiation of trophoblast cells into particular mature cell types, and of inducing proliferation of endothelial cells (Adelman et al., 2000; Damert et al., 1997; Levy et al., 2000; Schaffer et al., 2003). Furthermore, deletion of key components of the cellular responses to hypoxia result in defects in placental development (Adelman et al., 1999, 2000; Kozak et al., 1997; Maltepe et al., 1997). *HIF1β* mutant placentas have an underdeveloped labyrinthine layer, and a disproportionate number of spongiotrophoblasts and TGC (Adelman et al., 2000). In vitro studies show that hypoxia normally biases differentiation of TS cells into spongiotrophoblasts instead of TGC, but TS cells derived from *HIF1β* mutants fail to show this increase in spongiotrophoblast differentiation in response to hypoxia (Adelman et al., 2000; Tanaka et al., 1998). It therefore seems likely that an aberrant response to cellular hypoxia alters cell fate in *HIF1β* mutant placentas, with severe impacts on placental development. It is unclear which HIF1-responsive gene(s) are required to direct trophoblast cell fate under conditions of cellular hypoxia, or how this is achieved at the molecular level. Moreover, hypoxia-independent roles for HIF heterodimers in the regulation of trophoblast differentiation have also recently been identified (Maltepe et al., 2005). We show here that null mutants for

Table 2
The phenotype of *Cited1/Cited2* compound nulls is remarkably similar to that of *Cited2* nulls at 14.5 dpc

Genotype	Weight (g) [a]		
	Embryo	Placenta	Ratio [b]
Cited1+/Y;			
Cited2+/+ (*n* = 8)	0.265 ± 0.011	0.111 ± 0.004	2.384 ± 0.096
Cited2+/− (*n* = 14)	0.236 ± 0.009*	0.106 ± 0.003	2.230 ± 0.095
Cited2−/− (*n* = 10)	0.221 ± 0.011*	0.076 ± 0.002*	2.924 ± 0.151*
Cited1+/−;			
Cited2+/+ (*n* = 10)	0.236 ± 0.015	0.111 ± 0.006	2.161 ± 0.147
Cited2+/− (*n* = 13)	0.225 ± 0.008	0.099 ± 0.006*	2.321 ± 0.087
Cited2−/− (*n* = 6)	0.200 ± 0.030*	0.063 ± 0.003*	3.169 ± 0.084*
Cited1−/Y;			
Cited2+/+ (*n* = 10)	0.240 ± 0.008	0.110 ± 0.005	2.203 ± 0.040
Cited2+/− (*n* = 11)	0.244 ± 0.011	0.099 ± 0.003	2.482 ± 0.136
Cited2−/− (*n* = 2)	0.185 ± 0.026*	0.066 ± 0.002*	2.781 ± 0.316*
Cited1−/−;			
Cited2+/+ (*n* = 6)	0.216 ± 0.008	0.087 ± 0.007#	2.541 ± 0.166
Cited2 +/− (*n* = 25)	0.223 ± 0.006	0.091 ± 0.003	2.463 ± 0.061
Cited2−/− (*n* = 4)	0.216 ± 0.021	0.064 ± 0.003*	3.342 ± 0.253*

Within each *Cited1* genotype (+/Y, +/−, −/Y, −/−) comparisons were made between individuals with different *Cited2* genotypes (+/+, +/−, −/−), using Student's *t* test, * ($P < 0.05$). Between each *Cited1* genotype (+/Y, +/−, −/Y, −/−) comparisons were made within individual *Cited2* genotypes (+/+, +/−, −/−), no statistical differences were observed.

[a] Mean ± standard error of the mean.

[b] Embryo/placenta ratio.

Cited2, a HIF1-responsive gene, develop placental defects similar to *HIF1β* mutant placentas, although less severe. Placentas were examined for the location and prevalence of hypoxic cells. Placentas lacking *Cited2* contained fewer hypoxic cells but this may have been due to the reduction in spongiotrophoblasts, TGC, and glycogen cells which are the cell types that we identified as having low oxygen levels. The best way of ascertaining if *Cited2* affects trophoblast differentiation through a role in the hypoxic response will be to examine trophoblast differentiation in the absence of *Cited2* under, physiologically relevant, low oxygen conditions.

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