Placental and fetal characteristics of the Ohia mouse line recapitulate outcomes in human hypoplastic left heart syndrome

Rebecca L Wilson[1], Weston Troja[1], Jennifer Courtney[1], Helen N Jones[1]*

[1]Center for Fetal and Placental Research, Cincinnati Children's Hospital and Medical Center, Cincinnati, Ohio USA 45229

*Corresponding author: Helen.Jones@cchmc.org
Cincinnati Children's Hospital and Medical Center
3333 Burnet Ave, Cincinnati Ohio USA 45229

Abstract

Congenital heart defects (CHDs) are one of the most common birth defects worldwide. The morbidity and mortality associated with these defects is compounded by increased frequency of fetal growth abnormalities in the newborns. Inappropriate placental development and function has been implicated as a contributing factor to poor fetal growth in pregnancies complicated by CHDs however, the exact mechanisms are poorly understood. In the Ohia mouse model of hypoplastic left heart syndrome (HLHS), the double homozygous genotype had previously been shown to be embryonically lethal at mid-pregnancy; a time in which optimal establishment of the placenta is crucial to fetal survival. We aimed to characterize placental and fetal growth and development in the double heterozygous genotype to determine whether the genetic mutations associated with HLHS in the Ohia mouse also affect the placenta. The frequency of fetuses with reduced weight near term was shifted in the double heterozygous genotype compared to wildtype fetuses. This shift in fetal weight distribution was associated with reduced fetal capillary density in the placentas of the double heterozygotes as well as a reduction in placental mRNA expression of angiogenic factors placenta growth factor (*Pgf*) and fms-like tyrosine kinase-1 (*Flt1*) suggesting abhorrent placental angiogenesis. Positive correlations were observed between fetal weight and placenta mRNA expression of several nutrient transporters in the double heterozygous genotype but not observed in the wildtype. This data shows changes to placental angiogenesis and nutrient transport that are likely to contribute to inadequate fetal growth in the Ohia mouse model. Such differences are similar to findings in studies of human placentas from pregnancies with a fetus with HLHS and highlights the importance of this mouse model in continuing to understand the link between placental development and CHDs such as HLHS.

New and Noteworthy

We used the Ohia mouse line, which is characterized with a hypoplastic left heart syndrome (HLHS)-like phenotype to investigate the contribution of placental development and function to fetal growth abnormalities associated with congenital heart defects (CHDs). We demonstrate an increase in the frequency of fetuses with lower fetal weight in the double heterozygous genotype which is associated with abnormalities to the placental microstructure, reduced placental fetal capillary density and placental mRNA expression of angiogenic factors *Pgf* and *Flt1*. These results are supported by similar studies of human placentas from HLHS pregnancies and highlights the usefulness of this model in furthering our understanding of abnormal fetal growth in CHDs.

Keywords: Hypoplastic left heart syndrome, congenital heart defects, placenta, Ohia mouse, fetal growth

Background

Congenital Heart Defects (CHDs) are the most common birth defect in the Unites States. The high risk of morbidity and mortality associated with CHD is compounded by increased frequency of low birth weight (LBW; <2500 grams), small for gestational age (SGA; <10th percentile), and microcephaly (<3rd percentile) in these newborns (1). One such defect is Hypoplastic Left Heart Syndrome (HLHS) and is characterized by the inability of the left ventricle to provide adequate systemic circulation (19) and is fatal shortly after birth without surgical intervention. The causes of HLHS remain unknown, but considerable evidence has demonstrated both genetic and environmental factors (6, 9). Newborns with HLHS are critically ill and require multiple reconstructive surgeries early in life. In addition to brain abnormalities, which occur in approximately half of newborns with HLHS, growth abnormalities at birth are common (2, 8). LBW and SGA are major risk factors for stage I Norwood palliative surgery, death and major complications following stage I palliative surgery (7, 10).

Currently, there is no clear link between fetal growth abnormalities and HLHS; however, fetal genetic factors, maternal health conditions and placental abnormalities have been implicated as contributing factors. The placenta has numerous functions that are crucial to supporting normal fetal growth including nutrient exchange for the developing fetus (12). Thus, abnormal placentation, development and function can negatively impact fetal growth leading to fetal growth restriction (FGR). The paucity of knowledge regarding the causes of FGR in pregnancies with fetuses that develop HLHS is being mitigated by the generation of the Ohia mouse model of HLHS (13). The HLHS-like phenotype in the Ohia mice arises from mutations in 2 genes: *Sap130* and *Pcdha9* with homozygotes being either embryonic lethal before or at mid-gestation, or rarely survive to late gestation and demonstrate severe cardiac defects in such as hypoplasia of the left ventricle, aorta and mitral valve (14).

The timing at which embryonic lethality occurs in the Ohia mouse model would suggest a significant impact on placental development. Placental development in the mouse can be divided into 2 broad stages (17, 22). The first stage of placental development last from implantation to mid-pregnancy and is characterized by trophoblast invasion and differentiation to form the mature mouse placenta. Fetal growth is supported by the yolk sac as there is no labyrinth to coordinate placental nutrient transport. The second stage is from mid-pregnancy onwards, where maternal blood flows through the mature placenta to support fetal growth. It is widely accepted that the switch at mid-pregnancy creates a developmental bottleneck leading to embryonic death in numerous mouse mutants (16, 22). In the Ohia mouse, extensive investigations have been conducted into heart development however, other aspects of fetal and placental development have not been investigated. Therefore, we aimed to determine whether the genetic mutations associated with HLHS in this mouse model also disrupt placental development and function.

Methods:

Mouse procedures:

C57Bl/6J-b2b635Clo/J (Ohia) mice were acquired from Jackson Laboratory (*Bar Harbor, ME*). All animal procedures were performed under protocol 2015-0087 and approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center. Animals were housed in individual cages (1-4 per cage), maintained in a 12-hour light/dark cycle, and food and water provided *ad libitum*. Ohia males double heterozygous for *Sap130* and *Pcdha9* ($Sap130^{m/+}Pcdha9^{m/+}$) were time mated with $Sap130^{m/+}$ $Pcdha9^{m/+}$ Ohia females for 12-hours overnight. At gestational day (GD) 18.5, females (n=4) were euthanized by CO_2 inhalation followed by cervical dislocation, and placentas and fetuses were weighed. Maternal organs, placentas, and fetal tissue were then either snap frozen or fixed in 10% formalin for genotyping and further analysis.

Genotyping:
Genomic DNA (gDNA) was extracted from fetal tails and adult ear clippings according to the HotSHOT protocol (20). Oligonucleotide primers were designed using Primer3 to amplify ~730bp regions surrounding *Sap130* and *Pcdha9* single nucleotide polymorphisms (SNPs) as identified by Liu et al. (14). Primer sequences were as follows: Sap130 (PCR) F: CCAGTGTGAAGTGAGTCAGCA, R: AGCACCATGTGCCTGTAATCT; Pcdha9 (PCR) F: CCTATGTTGATCGCCACTGC, R: CACCCTGAACACAAGTTCTATTGG; Sap130 (Sequencing) F: TTTTTACCCTCTTTTCAGGATCT, R: TCTGGGACACACATTTGTCAT; Pcdha9 (Sequencing) F: ACCTGATCATTGCCATTTGC, R: AAGTCAAATTCAGAGTTGCTT. gDNA regions were amplified via PCR using 1/150th gDNA and 300nM forward and reverse primers in a 50 µL FastStart PCR Master Mix reaction (Roche). PCR products were purified using QIAquick PCR Purification Kit (*Qiagen*). Purity and concentration was determined using agarose gel electrophoresis. To assess SNPs, 30 ng of purified PCR product and 10 pmols of forward and reverse sequencing primers in a 12 µl reaction were submitted to the DNA Core at Cincinnati Children's Hospital Medical Center for Sanger sequencing. SNPs for *Sap130* and *Pcdha9* were confirmed using FinchTV (*Geospiza Inc*).

Placental Histology and Immunohistochemistry:
Placental tissues were fixed in 10% formalin for 24 hours at 4°C immediately after dissection. Tissues were embedded in paraffin, cut into 5µm serial sections, deparaffinized in xylene, and rehydrated. To assess physical structure of Ohia placentas, sections were stained with Hematoxylin and Eosin (H&E) following standard protocol. To analyze microstructure, immunohistochemistry (IHC) was performed to identify fetal vessels. Sections were incubated in 1X sodium citrate (pH 6.0) target retrieval solution (*Dako*) at 95°C for 30 minutes, followed by a 20 minutes at room temperature. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide, followed by incubation with a 10% goat serum and 1% BSA protein block to limit non-specific antibody binding. Anti-CD31 (ab28364, *Abcam*) was diluted to 1:100 and applied to the sections overnight at 4°C. Antibody binding was then amplified by incubating with goat anti-rabbit secondary antibody (1:200, *Vector Laboratories*), followed by incubation with the ABC reagent (*Vector Laboratories*), and visualized using DAB (*Dako*). Sections were counterstained with hematoxylin, dehydrated, and mounted. Histological examination was conducted by light microscopy using Nikon 80i microscope. Fetal capillary density was analyzed using a 36-L-Mertz grid as described (21).

Placental Gene Expression
Total RNA was isolated from snap-frozen whole placentas using the FastPrep-24 Classic bead beater homogenizer (*MP Biomedicals*) and the RNAeasy Mini Kit (*Qiagen*) per manufacturer's protocol. 1 µg of total RNA was reverse transcribed into cDNA according to the High Capacity cDNA Reverse Transcription kit protocol (*Applied Biosystems*). For angiogenic factor and nutrient transport analysis, oligonucleotide primers for SYBR Green assays were designed to span intron/exon boundaries, aligned against murine genome via Primer-BLAST to ensure specificity. Primer sequences are as followed: *Ang1* F: GCACGAAGGATGCTGATAAC, R: AACCACCAACCTCCTGTTAG; *Ang2* F: GCACAAAGGATTCGGACAAT, R: AAGGACCACATGCGTCAAA; *Vegfα* F: TTAAACGAACGTACTTGCAGATG, R: AGAGGTCTGGTTCCCGAA; *Pgf* F: GACCTATTCTGGAGACGACA, R: GGTTCCTCAGTCTGTGAGTT; *Kdr* F: CGTTGTACAAATGTGAAGC, R: CACAGTAATTTCAGGACCC; Flt1 F: TGACGGTCATAGAAGGAACA, R: TAGTTGGGATAGGGAGCCA; *Slc2A1* F: TGTGCTCATGACCATCGC, R: AAGGCCACAAAGCCAAAGAT; *Slc2A3* F: CATTGTCCTCCAGCTGTCTC, R: GCGTCCTTGAAGATTCCTGT; Slc38A1 F: GGACGGAGATAAAGGCACTC, R: CAGAGGGATGCTGATCAAGG; *Slc38A2* F: TCCTTGGGCTTTCTTATGCC, R: TTGACACGAACGTCAAGAGA; *Slc7A5* F: ATTCAAGAAGCCTGAGCTGG, R: GCAGGCCAGGATAAAGAACA; Slc7A8 F: AAGAAAGAGATCGGATTGGT, R: TTTCGGTGAGACAAAGATTC; *Rps20* F: GCTGGAGAAGGTTTGTGCG, R:

AGTGATTCTCAAAGTCTTGGTAGGC. PCR reactions were then performed using 300 nM reverse and forward primers, 1/40th of the cDNA template in a 25 µL reaction of SYBR Green PCR Master Mix. Gene expression assays were completed in duplicate and normalized to murine *Rps20* using Applied Biosystems StepOne-Plus Real-Time PCR System. Relative expression was calculated using the Comparative Ct (ΔΔCt) method.

Statistics and data presentation:

Data was graphed and analyzed statistically using Prism (v8.0.1). Unpaired t-test was used to compare all data except qPCR data in which Mann-Whitney test was used. Fetal and placental weight distributions were calculated using a Gaussian non-linear model. Correlations between placenta nutrient transporter mRNA expression and fetal weight were calculated using Spearman's method. All data are represented as mean + SEM except qPCR which is represented as median + interquartile range and deemed significant with $P<0.05$.

Results:

Fetal weight distribution is left-shifted in the Ohia heterozygote and associated with reduced fetal capillary volume density

No double homozygote mice were recovered at GD18.5 and therefore, no placentas could be assessed. Mean fetal weight remained at wildtype levels in the double heterozygote genotype (Figure 1A) however, fetal weight distribution was shifted towards lower fetal weights (Figure 1B). Within the double heterozygous genotype, Mean placental weight as well as placental weight distribution was similar between the wildtype and double heterozygotes (Figure 1C and 1D). Placental morphology was highly variable between double heterozygote genotypes but demonstrated abnormal architecture in comparison to wildtype littermates (Figure 2A) and there was a reduction in fetal capillary density in the double heterozygote placentas (Figure 2B and 2C). However, positive correlations between fetal weight and placenta mRNA expression of several nutrient transporters were observed in the double heterozygous genotypes but not in the wildtype (Table 1).

Table 1. Correlation between fetal weight and mRNA expression of nutrient transporters in the placenta of wildtype (*Sap130*$^{+/+}$ *Pcdha9*$^{+/+}$) and double heterozygous (*Sap130*$^{m/+}$ *Pcdha9*$^{m/+}$) Ohia mice

	Sap130$^{+/+}$ *Pcdha9*$^{+/+}$	*Sap130*$^{m/+}$ *Pcdha9*$^{m/+}$
	Spearman r value	Spearman r value
Slc2A1	-0.0857	0.643
Slc2A3	0.429	0.679
Slc38A1	0.543	0.321
Slc38A2	0.0857	0.643
Slc7A5	-0.200	0.607
Slc7A8	-0.200	0.789

Reduced placental capillary volume density was associated with reduced placenta mRNA expression of angiogenic factors Pgf and sFlt1

Changes in placenta microstructure of the Ohia mouse suggested reduced fetal angiogenesis. Compared to wildtype genotype, placenta mRNA expression of placenta growth factor (*Pgf*) and fms-like tyrosine kinase-1 (*Flt1* or vascular endothelial growth factor receptor 1 (*VegfR1*)) was reduced in the double heterozygotes (Figure 3A and 3B). There was no difference in the expression of angiopoietin 1 (*Ang1*) and 2 (*Ang2*), *Vegfα*, nor VegfR2 (*Kdr;* kinase insert domain receptor) between the different genotypes (Figure 3C-F).

Discussion

In humans, CHDs such as HLHS are often associated with poor fetal growth that is likely contributed to by inappropriate placental development and function. In the present study, we characterized fetal and placental development in the Ohia mouse line, which is characterized with a HLHS-like phenotype. Most notably, no double homozygote fetuses were recovered at GD18.5, an observation possibly explained in part, by placentation failure which has been shown to significantly contribute to embryonic lethality (16). Furthermore, there was a shifted frequency towards lower fetal weight in double heterozygotes that was associated with changes to the placenta microstructure. Normally, the anatomy of the placenta is highly vascular with complex villous structures that optimize nutrient, oxygen and waste exchange. Positive correlations between nutrient transporter mRNA expression and fetal weight observed in the double heterozygotes, which was not observed in the wildtype genotype, would suggest that the shift toward lower fetal weights was not due to inadequate nutrient transport. This finding, a possible compensatory response, as well as the potential influence of the maternal genotype which was also double heterozygous for the *Sap130* and *Pcdh9* genes, may explain why there was no difference in mean fetal weight between wildtype and double heterozygotes.

Several studies in human pregnancies in which the fetuses have HLHS have shown using ultrasound, normal umbilical artery blood flow (3, 8, 15) suggesting FGR is not due to abnormal placental blood flow and may be due to placental micro-pathology. A reduction in fetal capillary density in the placentas of the double heterozygotes, as well as reduced mRNA expression of *Pgf* and *Flt1*, would suggest abnormal placental angiogenesis. These results are supportive of outcomes in human placentas from pregnancies in which the fetus developed HLHS that show reduced mRNA expression of *PGF*, reduced protein expression of FLT1, and abnormalities in both the syncytiotrophoblast (multinucleated outer cell layer of the placental villi) and the villous vasculature (11). In terms of placenta angiogenesis, PGF can modulate non-branching angiogenesis through FLT1 (4). Data suggests that FLT1 is the more important driver as *Flt*$^{-/-}$ mice are embryonically lethal due to the inability of endothelial cells to form organized vascular channels (5) whereas *Pgf*$^{-/-}$ mice are born healthy with only minor changes to spiral artery remodeling and early placenta establishment (18). Never-the-less, reduced expression of both *Pgf* and *Flt1* in the double heterozygous Ohia mouse would indicate an imbalance in the angiogenic mechanisms within the placental consistent with the findings relating to reduced fetal capillary density and increased frequency of lower fetal weights.

The aim of the current study was to assess placental development and the impact on fetal growth in the Ohia mouse model which is characterized by the development of fetal cardiac defects similar to HLHS in humans. No fetuses with a double homozygous phenotype were found at GD18.5; possibly due to early placental failure that requires further investigation. This hypothesis is supported by a shifted fetal weight distribution towards lower fetal weight in fetuses that carried the double heterozygous genotype indicating perturbations in placenta function associated with the genetic mutations. The shifted fetal weight distribution was associated with reduced density of fetal capillaries in the placenta and reduced placental mRNA expression of *Pgf* and *Flt1* which reflect similar outcomes in human cases of HLHS. Overall, whilst the double heterozygous fetuses do not carry heart defects, placental development and function is impaired; an outcome likely to be worse with the double homozygous genotype which do have a heart defect. As such, our study highlights the usefulness of this model to continuing our understanding of the concurrent disruption of the placenta in Congenital Heart Defects such as HLHS and in particular, determining which is more important, the placenta, the heart or both?

Abbreviations

CHD: congenital heart defect

HLHS: hypoplastic left heart syndrome
PGF: placenta growth factor
FLT-1: fms-like tyrosine kinase-1
LBW: low birth weight
SGA: small for gestational age
FGR: fetal growth restriction
ANG1: angiopoietin 1
ANG2: angiopoietin 2
VEGF: vascular endothelial growth factor
VEGFR1: vascular endothelial growth factor receptor 1
KDR: kinase insert domain receptor

Declarations

Ethic Approval: All animal procedures were performed under protocol 2015-0087 and approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Consent for publication: All authors consent for publication

Availability of data and materials: Not applicable

Competing Interests: The authors declare no conflicts of interest

Funding: This work was funded by the Cincinnati Children's Hospital and Medical Center Fetal Research Gift Fund

Authors contributions: RLW analyzed the data and drafted the manuscript. WT and JC performed the experiments and edited the manuscript. HNJ conceived and designed the study and edited the manuscript. All authors approve the final version.

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Figure Legends

Figure 1. Fetal and placenta weight in Ohia mouse at GD18.5. Mean fetal weight was not different between double heterozygous (*Sap130*$^{m/+}$ *Pcdha9*$^{m/+}$) and wildtype (*Sap130*$^{+/+}$ *Pcdha9*$^{+/+}$)(**A**) however, there was a shift in fetal weight distribution toward lower fetal weight in the *Sap130*$^{m/+}$ *Pcdha9*$^{m/+}$ genotype (**B**). Neither mean placenta weight (**C**) nor placenta weight distribution (**D**) was different between the double heterozygous and wildtype genotypes. Data are mean + SEM, n=4 females (15 wildtype and 18 double heterozygous fetuses), analyzed using unpaired t-test

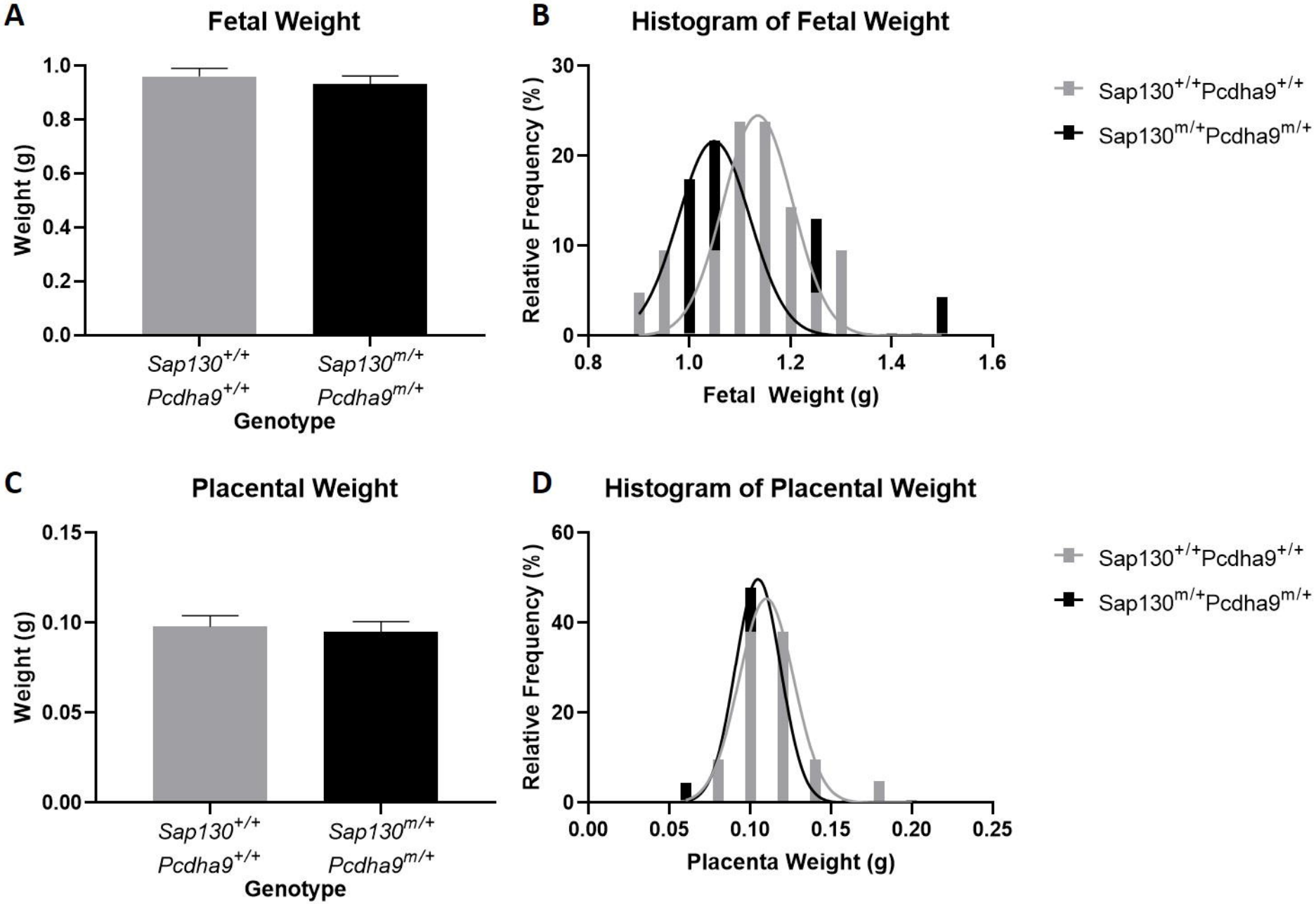
Figure 2. Placental microstructure of the Ohia mouse at GD18.5. Compared to the wildtype (*Sap130*$^{+/+}$ *Pcdha9*$^{+/+}$; representative image **A.1**) genotype, placental structure in the double heterozygous genotype (*Sap130*$^{m/+}$ *Pcdha9*$^{m/+}$; representative image **A.2**) was highly variable and exhibited abnormalities within the junctional and labyrinth regions. Further analysis found lower volume density of fetal capillaries in the placenta labyrinth of the double heterozygotes compared to wildtype (**B**) and shown in representative images **C.1** (wildtype) **C.2** (double heterozygous). Fetal capillaries were visualized using

immunohistochemistry against CD31 protein (stained in brown). Data are mean + SEM, n=3 females (6 wildtype and 5 double heterozygous), analyzed using unpaired t-test.

05

Figure 3. mRNA expression of angiogenic factors in the placenta of the Ohia mouse. Compared to wildtype (*Sap130*$^{+/+}$ *Pcdha9*$^{+/+}$), expression of placenta growth factor (*Pgf*; **A**) and fms-like tyrosine kinase-1 (*Flt1*; **B**) was reduced in the double heterozygous (*Sap130*$^{-/+}$ *Pcdha9*$^{-/+}$) placenta. There was no difference in the expression of angiopoietin 1 (*Ang 1*; **C**) or 2 (*Ang2*; **D**), vascular endothelial growth factor α (*Vegfα*; **E**) or VegfR2 (*Kdr,* kinase insert domain receptor; **F**). Data are median + interquartile range, n=3 females (6 wildtype and 7 double heterozygous placentas), analyzed using Mann-Whitney test

06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0.8
0.6
0.4
0.2
0.0
Sap130+/+ Pcdha9+/+
Sap130m/+ Pcdha9m/+
Genotype
B
Histogram of Fetal Weight
Relative Frequency (%)
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20
10
0
0.8
1.0
1.2
1.4
1.6
Fetal Weight (g)
Sap130+/+Pcdha9+/+
Sap130m/+Pcdha9m/+
C
Placental Weight
Weight (g)
0.15
0.10
0.05
0.00
Sap130+/+ Pcdha9+/+
Sap130m/+ Pcdha9m/+
Genotype
D
Histogram of Placental Weight
Relative Frequency (%)
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40
20
0
0.00
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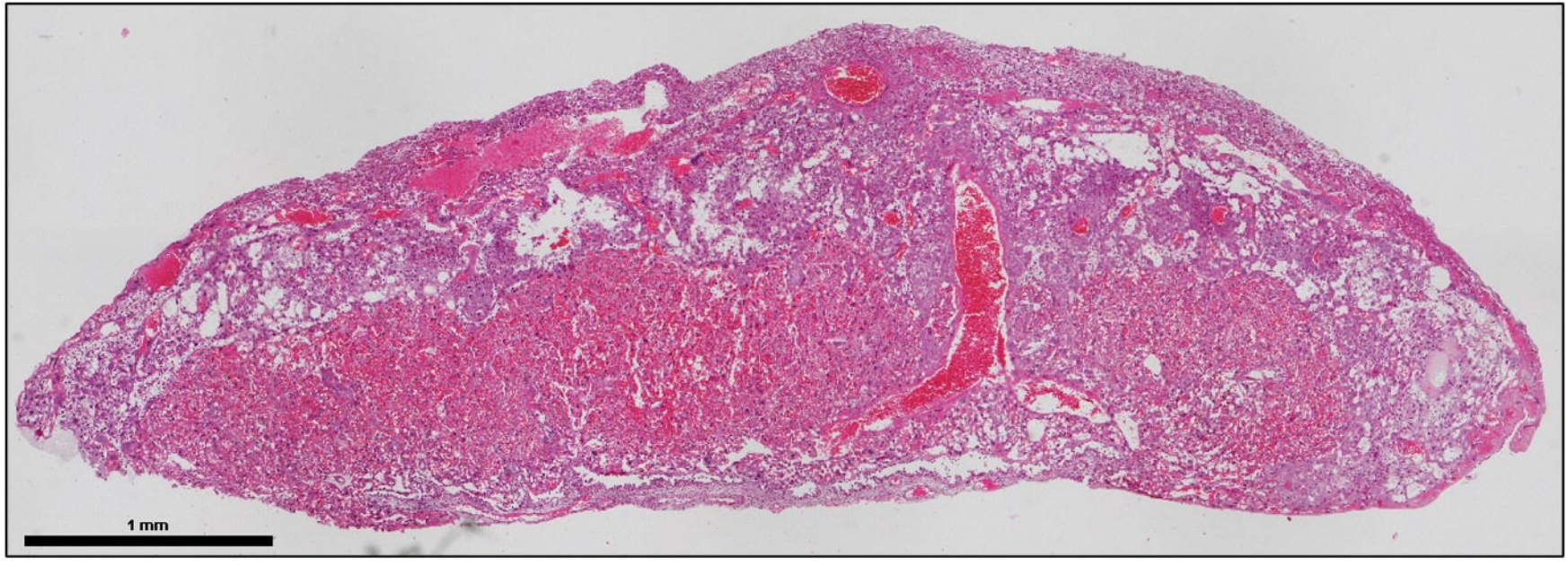
A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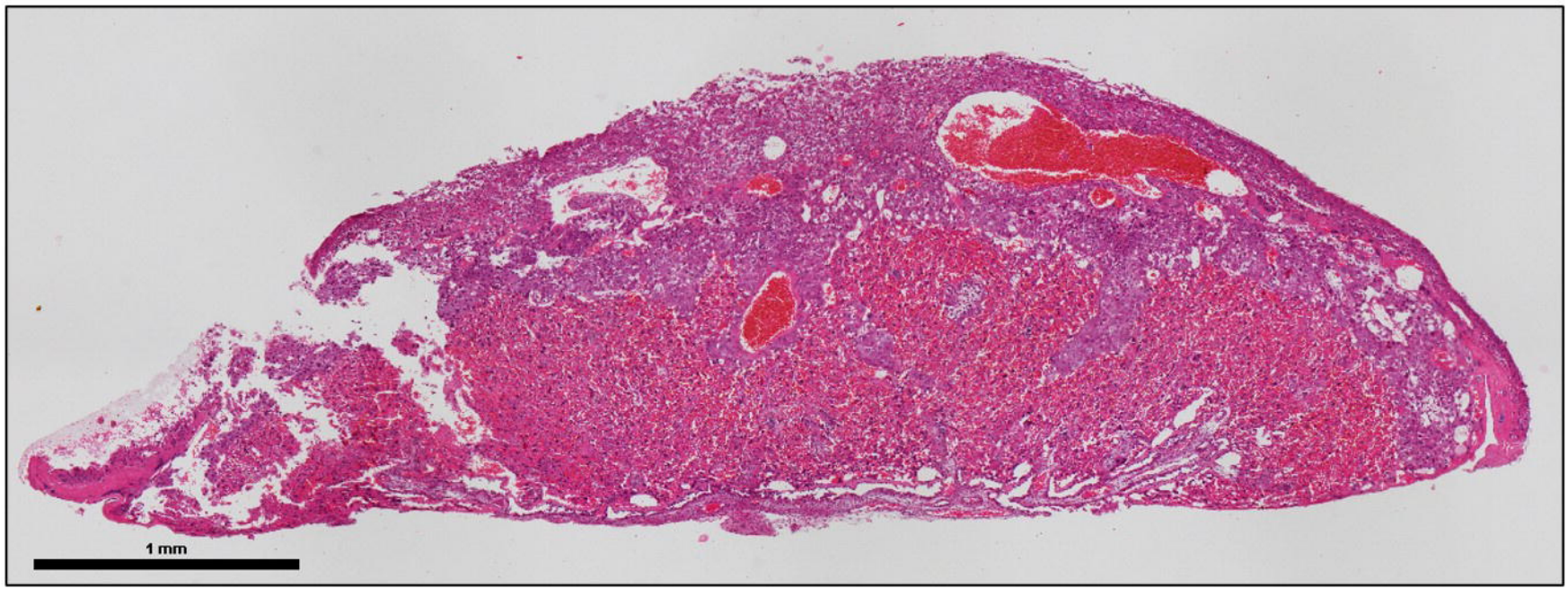
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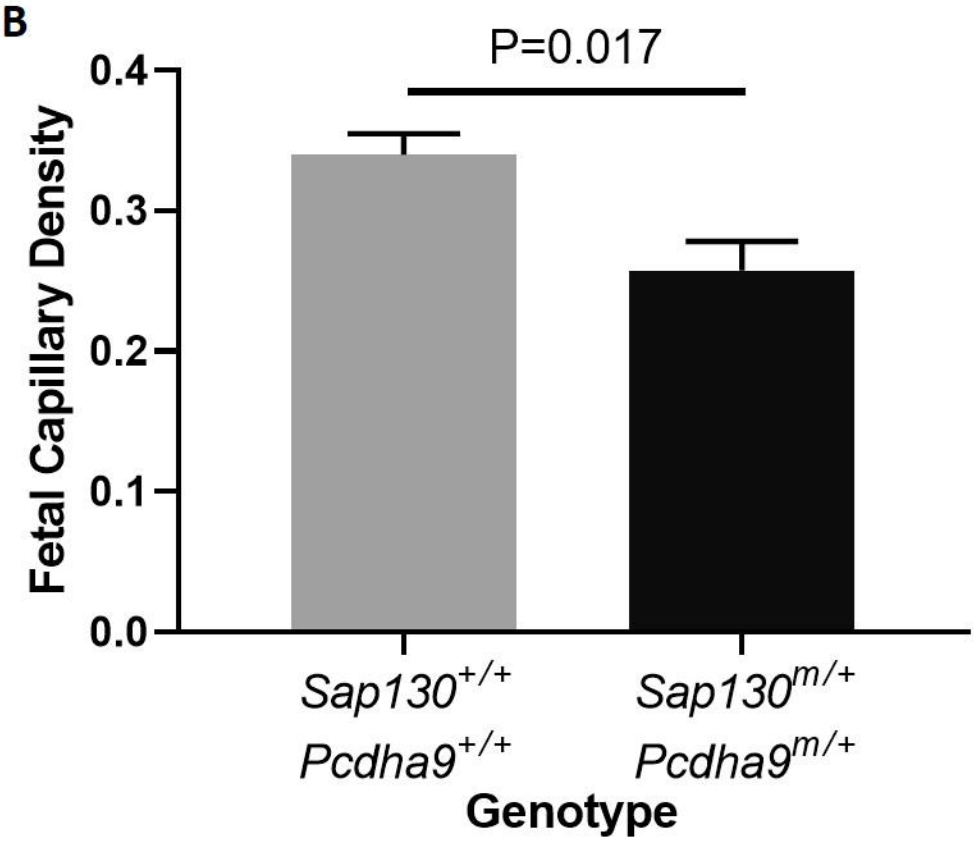
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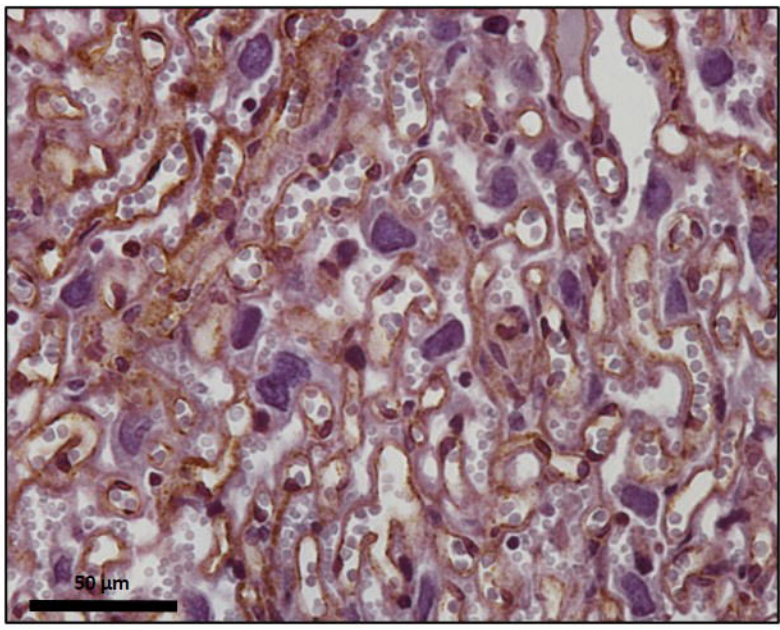
Genotype

C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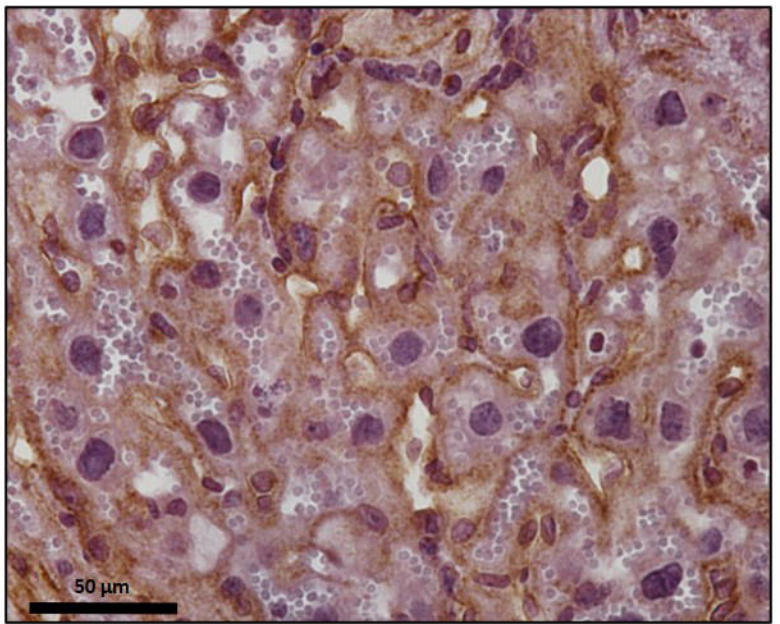
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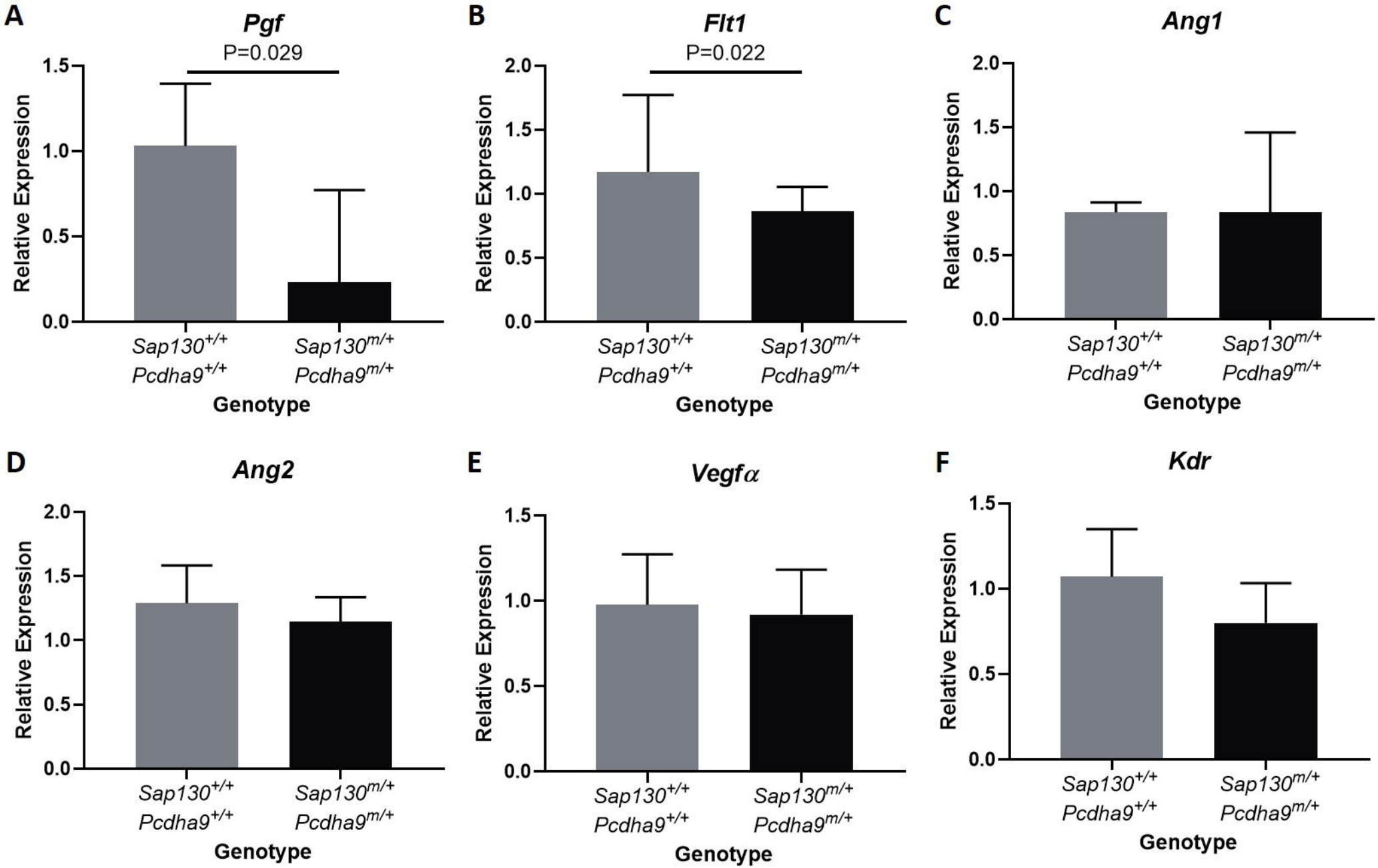
A
Pgf
P=0.029
Relative Expression
1.5
1.0
0.5
0.0
Sap130+/+ Pcdha9+/+
Sap130m/+ Pcdha9m/+
Genotype
B
Flt1
P=0.022
Relative Expression
2.0
1.5
1.0
0.5
0.0
Sap130+/+ Pcdha9+/+
Sap130m/+ Pcdha9m/+
Genotype
C
Ang1
Relative Expression
2.0
1.5
1.0
0.5
0.0
Sap130+/+ Pcdha9+/+
Sap130m/+ Pcdha9m/+
Genotype
D
Ang2
Relative Expression
2.0
1.5
1.0
0.5
0.0
Sap130+/+ Pcdha9+/+
Sap130m/+ Pcdha9m/+
Genotype
E
Vegfα
Relative Expression
1.5
1.0
0.5
0.0
Sap130+/+ Pcdha9+/+
Sap130m/+ Pcdha9m/+
Genotype
F
Kdr
Relative Expression
1.5
1.0
0.5
0.0
Sap130+/+ Pcdha9+/+
Sap130m/+ Pcdha9m/+
Genotype