



# *Klf14* is an imprinted transcription factor that regulates placental growth

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## ABSTRACT

**Introduction:** Imprinted genes are preferentially expressed from one parentally inherited allele, and many are crucial to the regulation of placental function and fetal growth. Murine Krüppel-like factor 14 (*Klf14*) is a maternally expressed imprinted transcription factor that is a component of the *Mest* imprinted gene cluster on mouse chromosome 6. We sought to determine if loss of *Klf14* expression alters the course of normal mouse extraembryonic development. We also used high-throughput RNA sequencing (RNAseq) to identify a set of differentially expressed genes (DEGs) in placentas with loss of *Klf14*.

**Methods:** We generated a *Klf14* knockout (*Klf14*<sup>null</sup>) mouse using recombineering and transgenic approaches. To identify DEGs in the mouse placenta we compared mRNA transcriptomes derived from 17.5dpc *Klf14*<sup>matKO</sup> and wild-type littermate placentas by RNAseq. Candidate DEGs were confirmed with quantitative reverse transcription PCR (qPCR) on an independent cohort of male and female gestational age matched *Klf14*<sup>matKO</sup> placentas.

**Results:** We found that 17.5dpc placentas inheriting a maternal null allele (*Klf14*<sup>matKO</sup>) had a modest overgrowth phenotype and a near complete ablation of *Klf14* expression. However, there was no effect on fetal growth. We identified 20 DEGs differentially expressed in *Klf14*<sup>matKO</sup> placentas by RNAseq, and subsequently validated five that are highly upregulated (*Begain*, *Col26a1*, *Fbln5*, *Gdf10*, and *Nell1*) by qPCR. The most enriched functional gene-networks included those classified as regulating cellular development and metabolism.

**Conclusion:** These results suggest that loss of the maternal *Klf14* locus in the mouse placenta acts results in changes in gene expression patterns that modulate placental growth.

## 1. Introduction

The metabolic and developmental processes controlling fetal and placental growth are strongly influenced by imprinted genes [1]. This remarkable set of genes is expressed exclusively or preferentially from one parentally inherited allele based on heritable DNA-methylation at gametic differentially methylated domains (DMDs). The widely accredited kinship theory postulates that misalignment of maternal and paternal selfish genetic reproductive interests contributes to a division in pro-growth paternally expressed and growth-limiting maternally expressed imprinted genes [2]. Murine Krüppel-like factor 14 (*Klf14*) is a maternally expressed imprinted gene encoding a transcription factor robustly expressed in the placental labyrinth [3].

Preferential expression of maternally inherited *Klf14* is dependent on

maternal parent-of-origin specific DNA methylation at the *Mest* DMD, which exerts transcriptional control of the paternally expressed *Mest* as well as a cluster of maternally expressed genes across a 400-kb region that includes *Copg2*, *Cpa4*, and *Klf14* [4,5]. Disruption of either the establishment or inheritance of imprinted DNA methylation results in loss of *Mest* DMD methylation *in utero* and a lack of *Klf14* expression within extraembryonic tissues [3,6]. Our previous work revealed that loss of imprinting at the *Mest* locus in late gestation is associated with reduced fetal-placental weight ratio [6], increased spongiotrophoblast central volume and elevated triglyceride levels [7,8]. We have interpreted these findings as strong evidence of a role for the *Mest* imprinting cluster in regulating placental growth and metabolism. This work is also in accordance with other studies linking the *Mest* imprinted locus to *in utero* placental and fetal development.

**Abbreviations:** DEG, Differentially expressed gene; DMD, Differentially methylated domain; eQTL, expressed quantitative trait locus; FDR, false discovery rate; *Klf14*, Krüppel-like factor 14; PAS, Periodic acid-Schiff; RNAseq, High-throughput RNA sequencing; RT-PCR, Reverse transcription PCR; qPCR, Quantitative RT-PCR.

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In late gestation (16.5–18.5dpc) in the mouse, an intrauterine and post-natal growth restriction phenotype is observed in fetuses with bimaternal inheritance of the subproximal 6q region encompassing the *Mest* and *Nap115* imprinting clusters, whereas fetal growth enhancement that resolved by term pregnancy is observed with bipaternal inheritance [9,10]. In humans, *matUPD7* and *Mest* DMD hypermethylation are causes of intrauterine growth restriction in a minority of Silver-Russell syndrome cases [11,12]. Together, this evidence suggests the imprinted (methylated) *Mest/MEST* maternal allele is integrated as a net growth restrictor during pre-natal development either through silencing of pro-growth paternally-expressed genes or maternal expression of growth restricting genes.

Multiple lines of evidence support a role for *Klf14* as a key metabolic transcriptional regulator. In humans, *KLF14* is a maternal-specific expressed quantitative trait locus (eQTL) associated with type-2 diabetes risk and HDL levels; variant polymorphisms rs731702 and rs972283 within the intergenic region upstream of *KLF14* result in diminished *KLF14* expression in *cis*, and altered downstream metabolic transcriptional networks in *trans* within adipocytes [13–15]. Recent work describing the function of *Klf14* in the mouse liver suggest a conserved role for *KLF14* in transcriptional regulation of metabolic homeostasis [16–18]. These effects are mediated by binding of *KLF14* to SP-1 like GC rich motifs in target gene promoters, where it acts predominantly as a transcriptional repressor [16–18]. While these studies in adipose and liver are informative, the tissue with the highest RNA expression of human *KLF14* is the placenta, corroborated by strong immunohistochemical staining in chorionic villi syncytiotrophoblast [19].

In this study, we sought to determine whether loss of *Klf14* expression alters the course of normal mouse extraembryonic development by examining placentas derived from a *Klf14* knockout mouse. Furthermore, because *Klf14* is a transcription factor, we pursued the identification of placenta-specific transcriptional changes by high-throughput RNA sequencing (RNAseq). We analyzed the differentially expressed gene sets to understand the pathways altered by loss of *Klf14*. Our work suggests that within the mouse placenta *Klf14* is an imprinted transcription factor that modulates expression of a defined set of genes that may have a role in placental growth.

## 2. Materials and methods

### 2.1. Generation of a *Klf14* null mouse

We employed recombineering technology to construct a targeting vector, and generate *Klf14<sup>lox</sup>* and *Klf14<sup>null</sup>* alleles (Fig. S1, Table S3). Recombineering utilizes bacterial strains with inducible gap-repair recombination enzymes that catalyze recombination between homologous 200–500 bp sequences (homology boxes) enabling efficient exchange of sequences from one vector to another (for reviews see Refs. [20,21]). A 13.5 kb region encompassing *Klf14* and surrounding sequences was retrieved from bMQ6044J02 bacterial artificial chromosome, obtained from Source Bioscience, and cloned into a plasmid adjacent to a diphtheria toxin gene. We then engineered plasmids to insert LNL and LFNTF cassettes to generate a targeting allele. Standard methods of ES cell transfection, homologous recombination, Flp electroporation, Neomycin/Ganciclovir positive/negative selection and blastocyst injection were used to generate a transgenic *Klf14<sup>lox</sup>* mouse line [22]. The *loxP* sites were thereby added approximately 1.6-kb upstream of the *Klf14* transcriptional start site and 300-bp downstream of the *Klf14* 3'-UTR separated by a total of 4.9-kb. This line was then crossed with *Sox2:CRE* transgenic females to generate a constitutive null allele (*Klf14<sup>null</sup>*).

### 2.2. Animal husbandry

Animals were cared for in accordance with the University of

Pittsburgh Institutional Animal Care and Use Committee. Heterozygous *Klf14<sup>null</sup>* mice were backcrossed 5 generations to the 129sv Taconic strain prior to setting up timed matings. We bred *Klf14<sup>hetKO</sup>* female mice to 129sv inbred wild-type mice to generate *Klf14<sup>matKO</sup>* and control offspring for phenotypic and transcriptional analysis. Additionally, control (*Klf14<sup>wt</sup>*), heterozygous (*Klf14<sup>hetKO</sup>*), and homozygous (*Klf14<sup>homKO</sup>*) derived from heterozygous intercrosses were studied for late gestation placental abnormalities.

### 2.3. Genotyping

Floxed alleles were confirmed by Southern blotting of *Bgl*III digests with an external 5' probe (*Klf14<sup>wt</sup>* band of 4.0-kb and *Klf14<sup>lox</sup>* band of 9.2-kb). Null alleles were distinguished from wild-type and floxed alleles by Southern blot of *Sca*I digested genomic DNA with an external 5' probe (*Klf14<sup>wt</sup>* and *Klf14<sup>lox</sup>* bands of 11.0-kb and *Klf14<sup>null</sup>* band of 6.1-kb). *Klf14* floxed, null and wild-type alleles were also genotyped by semi-nested PCR by the presence of 200-bp (*Klf14<sup>wt</sup>*), 300-bp (*Klf14<sup>lox</sup>*) and 400-bp (*Klf14<sup>null</sup>*) alleles (Table S3). *Sox2:CRE* transgene genotyping and *Zfy* sex-typing were also determined by PCR. Placental genotypes were ascertained from associated yolk-sac or fetal tissues.

### 2.4. Placental measurements

Late gestation (17.5dpc) mouse placentas were collected from crosses of heterozygous *Klf14<sup>null</sup>* females to either wild-type or heterozygous males. Placenta and fetal wet weights were recorded. Half of each placenta was cryo-preserved for histology. Nucleic acids were isolated from the remaining half for gene expression and methylation analysis. Placental layer fractions were determined using the ratio of the average spongiosotrophoblast, and the labyrinth area determined by random grid sampling of a single central placental section for each sample.

### 2.5. *Klf14* expression analysis

Nucleic acids were isolated using All-Prep or RNeasy kits (Qiagen) per manufacturer's instructions from fresh placental tissues, and other fetal and adult tissues isolated by microdissection. Prior to reverse transcription, RNA was treated with DNaseI for 1 h to remove genomic contaminants. First-strand cDNA synthesis was carried out with MMLV-RT (Promega) from 500 ng of RNA template using oligo (dT)<sub>18</sub> primers. Amplification of an 800-bp RT-PCR of *Klf14* cDNA product was performed using primers previously reported by Parker-Katirae et al. [3].

### 2.6. *Klf14* methylation analysis

The methylation status of the *Mest* DMD in wild-type and null placentas was examined by bisulfite genomic sequencing and combined bisulfite restriction analysis assays. Bisulfite genome conversion, PCR and Sanger sequencing of wild-type and *Klf14<sup>null</sup>* placental DNA was carried out as previously described using the same reagents and nested primers [6]. The *Bst*BI restriction enzyme was utilized to cut 1 µg of bisulfite converted *Mest* DMD PCR amplicon in order to examine for the presence of unconverted (methylated) sites within the *Mest* DMD.

### 2.7. Transcriptome analysis

Library preparation and high-throughput sequencing were performed at the Children's Hospital of Pittsburgh Genomics core. An Illumina TruSeq mRNA library preparation kit was used with indexing i5 and i7 adapters. Four control and four mutant samples were sequenced to a depth of 40 million reads on a NextSeq500 (Illumina) instrument. Computational analysis was performed on the high throughput computing cluster of the University of Pittsburgh Center for Research Computing. Quality control and trimming of sequencing adapters was

performed with Trimalore [23]. The reads were aligned to the ensembl v96 mouse genome and annotation using STAR v2.7 and gene counts tabulated using HTSeq [24,25]. Differential expression analysis was performed using the DESeq2 Bioconductor package [26]. Genome-wide transcription data can be found in the GEO Repository, Geo accession number GSE137918.

## 2.8. Quantitative RT-PCR

1 µg RNA isolated from placental tissues was subjected to DNaseI treatment (Ambion) and then used as template for first strand cDNA synthesis using superscript IV (Invitrogen). Amplification of target genes was performed using SYBR green mastermix on a 7900HT real time PCR machine (Applied biosystems). Primer sequences are given as supplemental (Table S3) and for lineage markers partly drawn from Tunster et al. [27,28].

## 2.9. Statistics

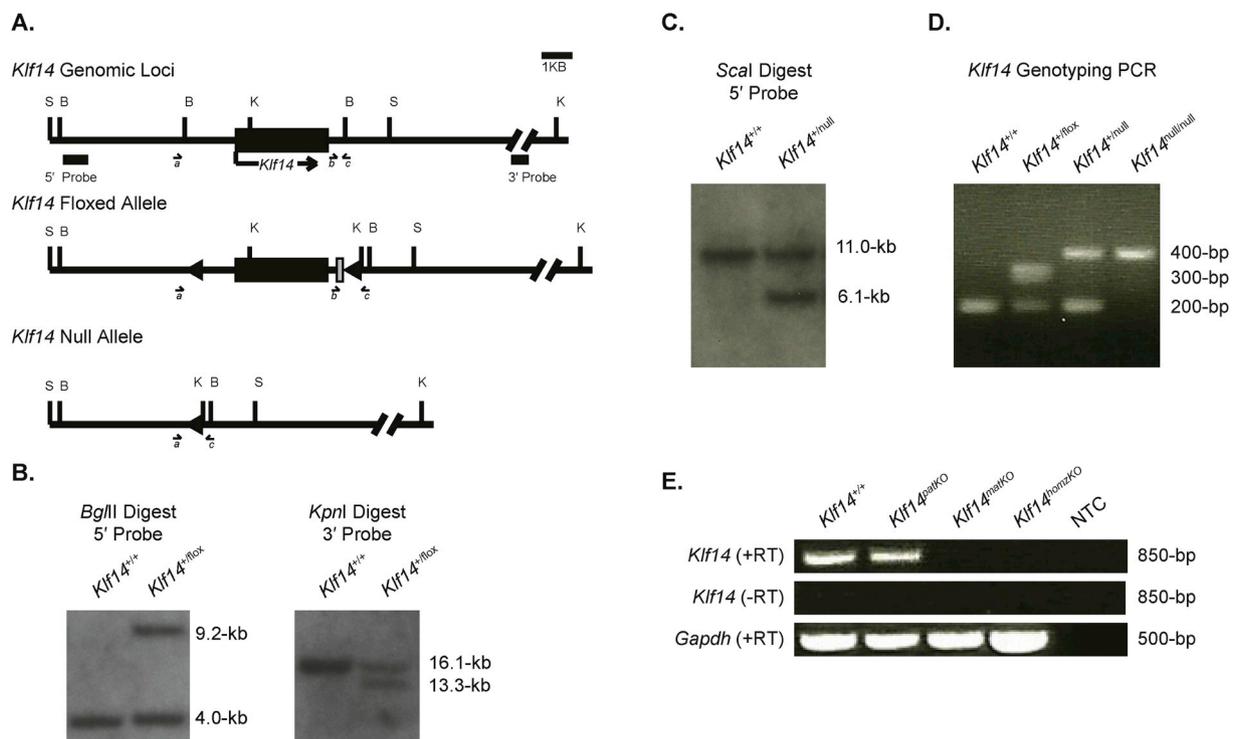
Morphometric comparisons were made between wild-type, heterozygous and null placentas by analysis of variance (ANOVA) followed by post-hoc Tukey-HSD. For qPCR data technical replicates were averaged and the ddCt method utilized to calculate the log<sub>2</sub> fold change between mutant and control groups separated by sex or aggregated as well as the standard deviation for each. Comparison across groups was made by either one-way (for DEGs) or two-way (for lineage markers) Welch's T-test with *p* value cutoffs at 0.05, 0.005 and 0.0005 for significance. A Benjamini-Hochberg false discovery rate (FDR) *p*-adjusted (*q* value) cutoff of *q* < 0.10 was used to identify candidate DEGs from RNAseq transcriptome analysis. A less stringent *p* value < 0.1 was used for a broader supplementary gene expression and gene network analysis via Ingenuity Pathway Analysis (Qiagen).

## 3. Results

### 3.1. Generation of a *Klf14* maternal knockout

We generated a floxed *Klf14* allele using homology directed mouse embryonic stem cell genome editing approaches to add *loxP* flanking the *Klf14* single exon open reading frame (Figs. 1A and S1). Analysis of transgenic mouse genomic DNA by Southern blotting confirmed the expected deletion of a *Bgl2* site in the targeted floxed allele at the *Klf14* upstream *loxP* site by the presence of a 5.2-kb larger *BglII* fragment detected by a dsDNA probe 5' of *Klf14* and external to the targeting construct (Fig. 1B). Floxed male mice were crossed with Sox2:CRE mice to generate constitutive *Klf14* null offspring. The deletion of a 4.9-kb region encompassing the single *Klf14* open reading frame and promoter sequence was distinguished by Southern blot of *ScaI* digested DNA, probed with the same 5' external probe (Fig. 1C). The wild-type, floxed and null alleles can also readily be distinguished by semi-nested genomic PCR (Fig. 1D).

A survey of *Klf14* mRNA expression in extraembryonic, fetal and adult wild-type tissues by reverse-transcription PCR (RT-PCR) revealed robust expression in yolk sac, placenta and fetal intestine with lesser expression in fetal brain, fetal liver, adult kidney and adult heart (Fig. S2). We then tested the degree of preferential maternal *Klf14* expression in placenta with inherited paternal null (*Klf14*<sup>patKO</sup>), maternal null (*Klf14*<sup>matKO</sup>) and homozygous null (*Klf14*<sup>homzKO</sup>) alleles. The placentas of *Klf14*<sup>patKO</sup> fetuses maintained wild-type levels of transcription. In contrast, expression of *Klf14* in *Klf14*<sup>matKO</sup> and *Klf14*<sup>homzKO</sup> placentas was undetectable (Fig. 1E). This result confirms strict maternal expression of *Klf14* in the mouse placenta and complete loss of expression when inheriting a maternal null allele. Additionally, we found there was no change in imprinted DNA methylation at the *Mest* DMD associated with inheritance of the *Klf14*<sup>null</sup> allele, suggesting that



**Fig. 1.** Genetic characterization of a novel *Klf14* floxed and constitutive null allele. A) Schematic of the native *Klf14* locus and genome edited floxed and null alleles. B) *BglII* restriction digest Southern blot of wild-type and heterozygous floxed mice. C) *ScaI* restriction digest and southern blot of wildtype and heterozygous null mice. D) Genomic genotyping semi-nested PCR identifies specific bands for wild-type (200-bp), floxed (300-bp) and null (400-bp) alleles. E) RT-PCR of an 850-bp *Klf14* cDNA amplicon in wild-type, *Klf14*<sup>patKO</sup>, *Klf14*<sup>matKO</sup> and *Klf14*<sup>homzKO</sup> placentas. Key: Filled rectangle, *Klf14* with arrow of 5'–3' transcription; directional triangles, *loxP* elements; grey rectangle, residual LFNTF vector sequence; Vertical lines indicate restriction sites (S) *ScaI*, (B)-*BglII*, (K) *KpnI*, (N) *NotI*; and a, b and c semi-nested PCR primer s. Scale bar is 1-kb.

there would be no *in cis* effects on the expression of neighboring imprinted genes within the subproximal 6q imprinted cluster (Fig. S3).

### 3.2. Placentas of *Klf14<sup>matKO</sup>* offspring are modestly overgrown in late gestation without overt morphological changes in placental layers

Consistent with other reports of *Klf14* constitutive null alleles, we found that both heterozygous (*Klf14<sup>hetKO</sup>*) and *Klf14<sup>homzKO</sup>* mice were viable and fertile [16,29]. Heterozygous intercrosses resulted in offspring that inherited the *Klf14<sup>null</sup>* allele at expected mendelian frequencies on both B6N and 129sv backgrounds (Chi square  $p > 0.05$ , Tables S1 and S2). A placental overgrowth phenotype was observed in late gestation placentas from *Klf14<sup>matKO</sup>* offspring compared to those of wild-type littermates (Fig. 2A; Tukey-HSD  $p = 0.0076$ ). However, although fetal weights in *Klf14<sup>matKO</sup>* offspring remained unchanged (Fig. 2B), there was no difference in fetal-placenta weight ratio. Similarly, placental but not fetal overgrowth was observed across genotypes of late gestation mouse placentas derived from *Klf14<sup>hetKO</sup>* 129sv intercrosses (Fig. S4; ANOVA  $p = 0.05$ ). Representative images of a *Klf14<sup>homzKO</sup>* and control littermate H&E and Periodic acid-Schiff (PAS) histological staining demonstrate a moderate overall increase in size in all layers with lack of any structural abnormalities and consistent glycogen content (Figs. 2C and S5). Likewise, there were no aberrations in the labyrinth to spongiotrophoblast placental layer fraction in *Klf14<sup>homzKO</sup>* placentas (Fig. S4). The expression of lineage markers was similar among the placental layers in *Klf14<sup>matKO</sup>* placentas, except for a slight reduction of the labyrinth marker *Gcm1* ( $p < 0.05$ ; Fig. S6).

### 3.3. *Klf14* regulates a placental transcriptional network including *Begain*, *Col26a1*, *Fbln5*, *Gdf10*, and *Nell1*

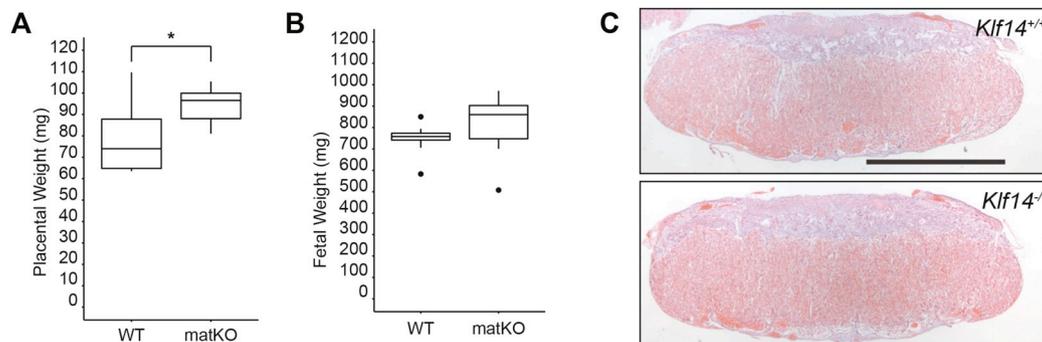
We sought to use *Klf14<sup>matKO</sup>* placentas to identify differentially expressed genes (DEGs) that could be mediating the overgrowth phenotype. Transcriptome wide gene expression profiling by RNAseq was performed on four *Klf14<sup>matKO</sup>* and four control 17.5dpc male placentas. The top 100 highest expressed genes in our RNAseq dataset (Table S4) overlapped considerably with known mouse placental labyrinth and spongiotrophoblast lineage markers and those highly expressed in rat placental compartments and were consistent across genotypes with abundant labyrinth and spongiotrophoblast transcripts and few from maternal decidual endometrium [30–32]. Expression of placental lineage markers used to examine layer composition by qPCR (Fig. S6), revealed similar consistent expression across control and *Klf14<sup>matKO</sup>* placentas with modest variability in labyrinth markers (Table S5) [27,28]. Not surprisingly, *Klf14<sup>matKO</sup>* placentas had a greater than 6 log2 fold decrease in *Klf14* expression (Table 1), but the expression of other imprinted and non-imprinted genes within the *Mest* imprinting cluster as well as the *Napl15* and *Peg10* imprinting clusters on

chromosome 6 were unchanged (Table S6). These results confirm strong, near absolute maternal parent-of-origin specific expression of *Klf14* in

**Table 1**

Table of differentially expressed genes identified in *Klf14<sup>matKO</sup>* placentas. Differential gene-level expression analysis of a comparison of four male matKO and four wild-type littermates, filtered (FDR  $q$  value  $< 0.10$ ) and arranged in ascending  $q$  value order. Normalized gene expression as Reads per Kilobase of transcript per Million reads (RPKM) averaged across control and matKO samples and differential expression magnitude as log<sub>2</sub> fold change (log<sub>2</sub> FC) for comparison.

Gene	Ensembl ID	Control RPKM	matKO RPKM	log <sub>2</sub> FC	FDR $q$ value
<i>Klf14</i>	ENSMUSG00000073209	431.6	5.3	-6.34	7.04E-39
<i>Col26a1</i>	ENSMUSG00000004415	20.2	87.6	2.12	2.65E-10
<i>Nell1</i>	ENSMUSG00000055409	24.7	106.5	2.11	1.16E-04
<i>Slit2</i>	ENSMUSG00000031558	124.1	260.9	1.07	1.36E-03
<i>Adcy2</i>	ENSMUSG00000021536	53.6	136.8	1.36	1.46E-03
<i>Gdf10</i>	ENSMUSG00000021943	15.7	94.9	2.60	4.29E-03
<i>Pkia</i>	ENSMUSG00000027499	45.8	103.2	1.18	4.29E-03
<i>Gm4779</i>	ENSMUSG00000045010	85.9	34.9	-1.30	1.19E-02
<i>Piezo2</i>	ENSMUSG00000041482	461.9	319.3	-0.53	1.19E-02
<i>Dkk2</i>	ENSMUSG00000028031	54.7	137.1	1.33	2.53E-02
<i>Naalad2</i>	ENSMUSG00000043943	48.2	91.0	0.92	4.10E-02
<i>Smarca1</i>	ENSMUSG00000031099	171.3	266.5	0.64	4.27E-02
<i>Cd163</i>	ENSMUSG00000008845	85.5	44.4	-0.96	4.95E-02
<i>Fbln5</i>	ENSMUSG00000021186	465.4	901.1	0.95	4.95E-02
<i>Matn2</i>	ENSMUSG00000022324	421.5	633.8	0.59	4.95E-02
<i>Begain</i>	ENSMUSG00000040867	13.0	66.4	2.36	4.95E-02
<i>Col8a1</i>	ENSMUSG00000068196	126.5	248.5	0.98	4.95E-02
<i>Raver2</i>	ENSMUSG00000035275	45.0	83.9	0.91	7.74E-02
<i>Tmem200a</i>	ENSMUSG00000049420	10.4	28.1	1.43	7.74E-02
<i>Igdc4</i>	ENSMUSG00000032816	105.7	211.4	1.00	7.82E-02
<i>Olfm2</i>	ENSMUSG00000032172	67.5	141.5	1.07	8.62E-02



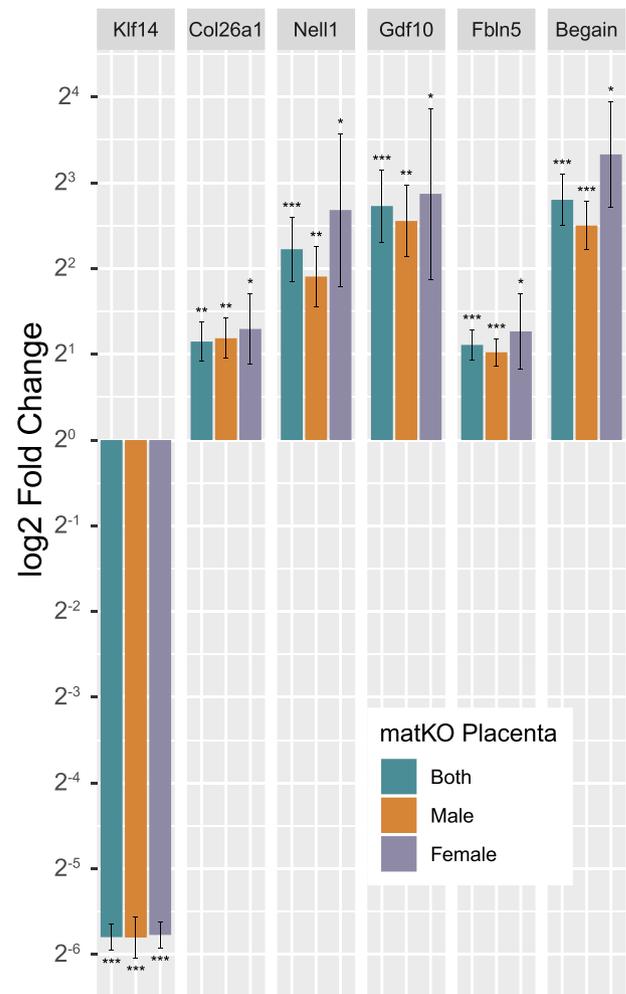
**Fig. 2.** Placental overgrowth in *Klf14<sup>matKO</sup>* offspring at 17.5dpc. **A)** Placental weights are increased in *Klf14<sup>matKO</sup>* placentas compared to wild-type littermates. **B)** Fetal weights remain unchanged between *Klf14<sup>matKO</sup>* and wild-type littermates. **C)** Representative H&E images of wild-type and homozygous 17.5dpc placentas (scale, 2 mm). WT, wild-type ( $n = 9$ ); matKO, *Klf14<sup>matKO</sup>* ( $n = 10$ ). Statistical comparisons by ANOVA and Tukey *post hoc* test,  $*p < 0.05$ .

the mouse placenta.

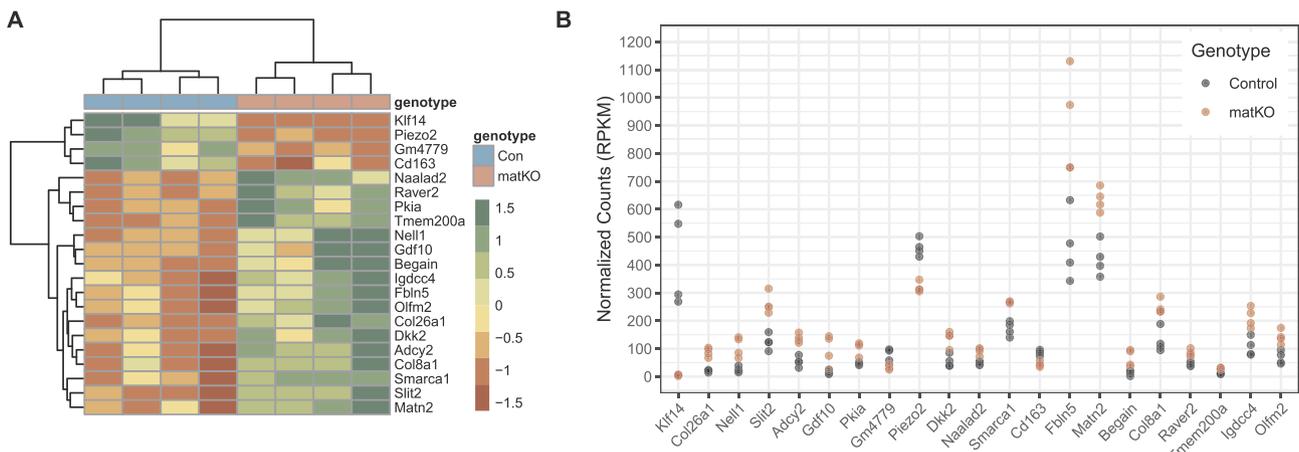
We identified 21 DEGs with an FDR  $q$  value less than 0.10 in *Klf14<sup>matKO</sup>* placentas (Table 1). The distribution of significant DEGs as shown by a volcano plot was skewed toward elevated expression, indicative of transcriptional de-repression (Fig. S8). Hierarchical clustering of the DEGs revealed two sets of transcripts (Fig. 3A). The first set was a small group of 4 genes with diminished expression including *Klf14*. The second set was a larger group of 17 genes with elevated expression. The normalized RNAseq read counts were consistent across samples of the same genotype for each identified DEG (Fig. 3B). These results identify a possible network of *trans* regulated genes repressed by *Klf14*.

To confirm changes in gene expression identified by RNAseq we analyzed additional cohorts of both male and female *Klf14<sup>matKO</sup>* 17.5dpc placentas and wild-type littermates by quantitative RT-PCR (qPCR). This analysis confirmed the elevated expression of *Begain*, *Col26a1*, *Fbln5*, *Gdf10*, and *Nell1* in *Klf14<sup>matKO</sup>* 17.5dpc placentas (Fig. 4). Results were largely concordant between male, female and combined cohorts with lesser statistical significance in female cohorts due to lower numbers of samples assayed. The discovery of *Gm14296*, encoding a putative zinc finger protein, as differentially expressed in the RNASeq data set was refuted by qPCR, although it was impossible to distinguish it from segmentally duplicated isoforms (Fig. S7). We were also able to verify changes in expression of *Col9a2*, *Fgg*, *Tlr12*, *Enpp2*, and *Ltp2* but not *Cp* which were identified using a less stringent unadjusted  $p$  value cutoff of less than 0.10 (Fig. S7, Table S7). Additionally, we tested the known hepatic *Klf14* targets *Sphk1* and *Pgc1a* but found no difference in expression (Fig. S7).

To better understand the network of DEGs in *Klf14<sup>matKO</sup>* placentas we performed Ingenuity Pathway Analysis (IPA). We discovered two enriched functional groups that included cellular development and growth as facets (Table 2). By superimposing these two overlapping networks we exposed a putative placenta cellular developmental regulome controlled by *Klf14* (Fig. S9). Additionally, we performed IPA functional analysis with a larger list of 525 genes identified using a less stringent non-adjusted  $p$  value cutoff of 0.1 (Tables S7–10). The larger analysis identified networks involved in cellular and organismal development, cardiovascular disease and metabolism among others (Table S8). We further identified additional canonical pathways and upstream regulators (Tables S9 and S10). These functional gene categories provide a potential molecular circuit that influences placental growth and development.



**Fig. 4.** qPCR validation of transcriptional changes in *Klf14* and DEGs. Log<sub>2</sub> fold change of six transcripts in independent control and matKO samples. Fold change was calculated by the ddCT method using *Gapdh* as the reference and WT sex-matched cohorts as 1.0 fold baseline. Male-WT n = 5, Male-matKO n = 7, Female-WT n = 5, Female-matKO n = 4. Mean fold change and SEM are shown. \* $p < 0.05$ , \*\* $p < 0.005$  and \*\*\* $p < 0.0005$ .



**Fig. 3.** Differential RNA-Seq expression analysis of 17.5dpc *Klf14<sup>matKO</sup>* placentas compared with wild-type littermates. Gene level RNA-Seq analysis of four *KLF14<sup>matKO</sup>* and four wild-type littermates. **A)** Heatmap clustergram of 21 DEGs including *Klf14* in *Klf14<sup>matKO</sup>* placentas. Green = upregulated, Red = down-regulated. **B)** Normalized RNAseq read counts for each of 21 DEGs including *Klf14*. Grey circle are WT samples and red circles are *Klf14<sup>matKO</sup>* samples. RPKM, reads per kilobase of transcript per million reads.

**Table 2**

Functional enrichment of differentially expressed genes in *Klf14<sup>matKO</sup>* placentas. The highest enriched pathways suggest an overlap in cellular growth and development.

Functional Category	Score	DEGs in Network
Cellular Development, Cellular Growth and Proliferation, Cellular Movement	28	<i>Begain, Cd163, Col8a1, Fbln5, Gdf10, Nell1, Olfm2, Pkia, Smarcal1, Tmem200a</i>
Cell Morphology, Cellular Assembly and Organization, Cellular Development	22	<i>Adcy2, Col26a1, Gm4479, Dkk2, Idgcc4, Naalad2, Piezo2, Raver2, Slit2</i>

## 4. Discussion

### 4.1. *Klf14* is a negative regulator of placental growth

Our results demonstrate that *Klf14<sup>matKO</sup>* placentas exhibit a modest placental overgrowth, which is reciprocal and in similar magnitude to the placental growth restriction seen in *Mest<sup>patKO</sup>* mice [33]. These findings suggest that *Mest* and *Klf14* influence placental growth in an antagonistic manner, congruent with the Kinship hypothesis with the paternally expressed *Mest* functioning to enhance placental growth and *Klf14* acting to limit it [2]. Our failure to observe an overt abnormal lipid accumulation in *Klf14<sup>matKO</sup>* placentas compared to the effect of concurrent loss of *Klf14* expression and gain of *Mest* expression [7] suggests the importance of combined imprinting of both genes and/or expression of other components of the *Mest* cluster. The overall impact of maternal *Klf14* deletion on placental growth was on par with maternal deletion of another imprinted gene, *Grb10* [34,35]. However, the *Klf14<sup>matKO</sup>* growth effect did not affect layer fractions, whereas *Grb10<sup>matKO</sup>* placentas had an increase in spongiotrophoblast at the expense of labyrinth [34,35]. Other imprinted genes, notably *Igf2*, have a greater effect on placental development with paternal deletion of placental *Igf2* (*Igf2p0*), somatic *Igf2* or the *H19* DMD having disrupted spongiotrophoblast and glycogen cell layers and pronounced placental growth effects [36]. Our work supports the hypothesis that imprinted genes have an integrated role in placental growth and metabolism with different clusters regulating specific processes.

### 4.2. Loss of *Klf14* modulates expression of a network of genes

Our findings indicate that loss of *Klf14* activates expression of multiple placental genes. These results are consistent with reported studies on KLF14 and its orthologs as repressors in both vertebrate and invertebrate species [37]. Our data are also consistent with KLF14's role in human adipocytes, where it has been implicated primarily as a transcriptional repressor, with 9/10 trans-eQTL genes upregulated concomitant with diminished *KLF14* expression associated with maternal inheritance of rs4731702 [13,15]. More recently, the *Klf14* eQTL studies have been expanded on to show that while the phenotypic associations with metabolic syndrome were female-specific the changes in gene expression were irrespective of sex [38]. However, none of the 20 DEGs identified in *Klf14<sup>matKO</sup>* placentas overlap the trans-eQTL targets nor those previously studied in targeted *Klf14* deletion mice [16–18, 38,39]. However we did find an overlap of 9 genes from an expanded 385 adipocyte trans-eQTL loci linked to rs4731702 [38], and our 525 less stringent cutoff DEG list (Table 7; data not shown). These discrepancies between studies identifying *Klf14* expression networks may be explained by differences in adipocyte, liver and placental transcriptional regulation.

Our IPA analysis indicated functional enrichment for genes involved in cellular growth and development (Tables 2 and S8). Canonical pathways identified from our larger list of candidate DEGs (Table S9) revealed links to sphingosine kinase-1 and polo-like kinases that have previously been identified as associated with *Klf14* [16,29], as well as activation of the ligand-activated nuclear receptors LXR and RXR,

known components of placental developmental and transcriptional metabolic networks [40–42]. Intriguingly TGFB is one of the highest scoring upstream regulatory node identified by IPA in our study (Table S10). This corroborates studies showing *Klf14* transcriptional activation by TGFB and subsequent attenuation of TGFBR2 activation via repression in Panc-1 cells, and TGFB/progesterone induced *Klf14* activation in Leydig cells [39,43]. The associated gene networks we have identified point towards an interplay between multiple pathways influenced by *Klf14*.

The set of DEGs identified herein is smaller than the 87 DEGs identified in *Klf14* adipose-specific knockout mice [38] and could be explained by the diverse tissue lineages in the placenta. Thus, we suggest that RNAseq of *Klf14<sup>matKO</sup>* of specific placental compartments, namely the labyrinth, may yield a larger group of trans-regulated genes. Additionally, within the placenta there is expression of redundant KLF paralogs that bind with similar affinities to SP1-like motifs. For example, the binding site for KLF6, the most highly expressed KLF in the mouse placenta, has been described *in vivo* as the SP1-like core GGCG motif at the promoter of genes coregulated by the placental developmental transcriptional regulators PPARG and LCoR [44]. This motif is encompassed by the predicted KLF14 binding site [45]. Moreover, the mouse *Klf6* KO leads to severe placental anatomical abnormalities. A combinatorial approach to deletions of *Klf* paralogs in addition to *Klf14* may lead to more severe morphological defects and transcriptional changes, and a larger DEG set.

In summary, our work shows that *Klf14* is an imprinted gene in the mouse placenta that when ablated results in placental overgrowth and transcriptional changes. Based on our findings of a unique network of disrupted genes in *Klf14<sup>matKO</sup>* placentas we suggest that *Klf14* has a discrete function in the placenta as a transcriptional regulator, and it influences the intrauterine environment by modulating pathways involved in placental growth.

## Declaration of competing interest

The authors have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.placenta.2019.09.013>.

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