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Original Research

COUP-TFI deletion affects angiogenesis and apoptosis related gene expression in mouse placenta: results of an explorative study

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Abstract

Background: Chicken Ovalbumin Upstream Promoter-Transcription Factor I (*COUP-TFI*) is a member of the steroid/thyroid nuclear receptor superfamily. The aim of this study was to investigate whether absence of this gene affects placental development and fetal growth in a *COUP-TFI* knockout mouse model. **Methods**: Placentas of *COUP-TFI*-knockout (*COUP-TFI KO*) and wild-type (*WT*) were collected at 18.5 days post-coitum. The expression level of the following genes known to be involved in different key molecular pathways was evaluated: BCL2 Associated X (*Bax*) and B-cell lymphoma 2 (*Bcl-2*) (apoptosis), *p21*, *p53* and α subunit of inhibin (*INHA*) (proliferation and apoptosis), vascular endothelial growth factor A (*VEGF-A)*, placental growth factor (*PlGF*), hypoxia-inducible factor 1-alpha (*HIF1*α), Fms related receptor tyrosine kinase 1 (*Flt-1*), and endoglin (*ENG*) (angiogenesis). Mouse litter weight at birth was also assessed. **Results**: RT-qPCR analysis showed increased mRNA expression of *VEGF-A* and *Bax* in placental tissue of *COUP-TFI KO* mice compared to *WT* mice. We also found a loss in the positive correlation between *Bcl-2* and *INHA*, *p21* and *ENG*, as well as *HIF1*α and *Flt-1* mRNA expression in *COUP-TFI* mutants. Finally, KO mice were lighter than WT littermates (respectively, the mean weight of *COUP-TFI KO* mice was 1.3 grams, $\pm$ 0.13, compared to 1.6 g, $\pm$ 0.14 of WT mice, $p < 0.05$). **Conclusions**: Our results show that *COUP-TFI* deletion is associated with a lower birth weight in mice and increased placental transcript expression of pro-apoptotic *Bax* and pro-angiogenetic *VEGF-A* genes.

Keywords: COUP Transcription Factor I; Nr2f1/NR2F1; Mouse; Placenta; Angiogenesis; Apoptosis

1. Introduction

At the basis of the development of a viable pregnancy in mammals there is the adhesion and implantation of a blastocyst to the endometrial epithelium. While the inner cell mass of the blastocyst will form the embryo, the outer layer, called the trophoblast, further develops into the maternal decidua and gives rise to the placenta. This latter represents the pivotal organ linking the developing embryo to the mother, providing the necessary oxygen supply and nutrient exchange [1]. Proliferation, apoptosis, and angiogenesis are crucial mechanisms involved in the correct development and remodeling of all the complex structures that make up the placenta. Impairments of this processes can result in several complications, including a deficient growth of ongoing pregnancy [2].

Chicken Ovalbumin Upstream Promoter-Transcription Factor I (*COUP-TFI*), also known as nuclear receptor subfamily 2, group F, member 1 (*NR2F1*) is an orphan nuclear receptor factor and member of the steroid/thyroid hormone receptor superfamily, mainly expressed in the central and peripheral nervous systems. The mammalian *COUP-TFI* plays a key role during metabolic homeostasis as well as organogenesis through cell fate determination, differentiation, proliferation, and apoptosis [3,4]. Overall, the high conservation of amino acid sequence between species suggests vital evolutionary conserved functions worth being explored, also when considering the process of placental development [5–7].

Earlier studies conducted on human placenta highlight the potential key role played by *COUP-TFI*. An *in-silico* investigation of transcriptional profile obtained using a microarray approach revealed that COUP-TFI was

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highly associated with self-renewal and differentiation of human chorionic trophoblast progenitor cells [8]. Furthermore, *COUP-TFI* was indicated in a meta-analysis among the genes that seems to play a role in the development of pre-eclampsia, a complication of placental function often related to fetal growth restriction [9]. However, whether and how *COUP-TFI* could influence the process of placental development has not been evaluated yet.

Compared to the monolayer in human placenta (hemomonochorial), placenta in mice is composed by three trophoblast layers (hemotrichorial) [10]. At embryological (E) day 10.5, when mid-gestation begins, all the layers of the placenta are formed, including the outermost maternal part, called the decidua, and the fetal part with the triple trophoblastic layer [11,12]. Embryonic development ends with the mid-gestation phase at E13.5, thereafter the fetus matures until the time of birth, around day E19.5 [13].

Transgenic *COUP-TFI KO* mice have been developed to shed more light into *COUP-TFI* functions [14]. Upon *COUP-TFI* loss, mice litter show a high incidence of perinatal mortality due to several neuronal malformations, particularly in the glossopharyngeal ganglion, defects in axonal arborization, and loss of cortical patterning due to the absence of thalamocortical connections [7,15].

The aim of this study was to explore whether and how *COUP-TFI* deletion in mice could interfere with placental development in terms of expression of some genes related to proliferation, apoptosis, and angiogenesis and in terms of neonatal weight at birth.

2. Materials and methods

2.1 Animals

COUP-TFI KO (COUP-TFI –/–) mice were generated and subsequently genotyped using the following primers, as previously described: forward 5′-CTGCTGTAGGAATCCTGTCTC-3′, reverse 5′-AATCCTCCTCGGTGAGAGTGG-3′ and reverse 5′-ACATACACAGCCTGGCCTTGC-3′ [14,16,17]. Heterozygous mice (COUP-TFI+/–) were bred together to generate *COUP-TFI KO* mice. Placentas were collected at 18.5 days post-coitum (dpc). The weight of pups was recorded at birth (post-natal day 0). Embryonic day (E) 0.5 was defined as the midday of the day of the vaginal plug. The study and all mouse experiments were conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and was approved by the local ethical committee in France (CIEPAL NCE/2019-548) [18]. One pregnant female at a time was euthanized by cervical dislocation and placentas were collected from foetus. Ten placentas were analyzed in this study; five of them were wild type and 5 were mutant (*COUP-TFI KO*). The placentas were separated from the surrounding tissue according to the previously described technique [19]. Subsequently, sagittal central sections of the placentas were used for the analysis [19].

2.2 Real-time PCR

All samples were kept on ice during dissection, then quickly transferred into 500 μL of TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA catalog number: 15596026) and processed for total *RNA* isolation according to the manufacturers' protocol. 1 μg of *RNA* was reverse transcribed using the SuperScript® III REV transcript Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA catalog number: 12574026). Quantitative *RT-PCR* reactions were performed by using Roche LightCycler® 480 and SSOADV UNIVER SYBR GRN SMX 500 (BIO-RAD, Hercules, California, USA catalog number: 172-5270), according to the manufacturers' protocols.

The following genes have been included as markers for placental development: hypoxia-inducible factor 1-alpha (*Hif1*α), endoglin (*ENG*), Fms related receptor tyrosine kinase 1 (*Flt1*), placental growth factor (*PlGF*) and the isoform A of the vascular endothelial growth factor (*VEGF-A*), being among the main players involved in angiogenesis pathway, *p21* and *p53* for cell proliferation and apoptosis, and BCL2 associated X (*Bax*), BCL2 apoptosis regulator (*Bcl2*), and α subunit of inhibin (*INHA*), involved in apoptosis and cell survival. The primers used are displayed in Table 1.

Table 1. List of used primer sequences.

Primer	Name
Sense 5′- CCGAGAATGGGAAGCTTGTC -3′	*Gapdh*
Antisense 5′-TCTCGTGGTTCACACCCATC -3′	*Gapdh*
Sense 5′- CCTTTTTGCTACAGGGTTTCATC -3′	*BAX*
Antisense 5′-AGCTCCATATTGCTGTCCAGTT -3′	*BAX*
Sense 5′- AAGCTGTCACAGAGGGGCTA -3′	*Bcl-2*
Antisense 5′-TCAGGCTGGAAGGAGAAGATG -3′	*Bcl-2*
Sense 5′- TGTCGCTGTCTTGCACTCTG -3′	*p21*
Antisense 5′-CCAATCTGCGCTTGGAGTGATA -3′	*p21*
Sense 5′- TGCTCACCCTGGCTAAAGTT -3′	*p53*
Antisense 5′-GTCCATGCAGTGAGGTGATG -3′	*p53*
Sense 5′- ATGAACTTTCTGCTCTCTTGGGT -3′	*VEGF-A*
Antisense 5′-CACAGGACGGCTTGAAGATGTA -3′	*VEGF-A*
Sense 5′- TGCTGGTCATGAAGCTGTTC -3′	*PlGF*
Antisense 5′-GGACACAGGACGGACTGAAT -3′	*PlGF*
Sense 5′- GACGATGAACATCAAGTCAGCA -3′	*HIF1*α
Antisense 5′-GGAATGGGTTCACAAATCAGCAC -3′	*HIF1*α
Sense 5′- GAGGAGGATGAGGGTGTCTATAG -3′	*Flt-1*
Antisense 5′-TGATCAGCTCCAGGTTTGACT -3′	*Flt-1*
Sense 5′- CTTCCAAGGACAGCCAAGAGT -3′	*ENG*
Antisense 5′-GTGGTTGCCATTCAAGTGTGG -3′	*ENG*
Sense 5′- TCGAAGACATGCCGTTGGG -3′	*INHA*
Antisense 5′-AGCTGGCTGGTCCTCACA -3′	*INHA*

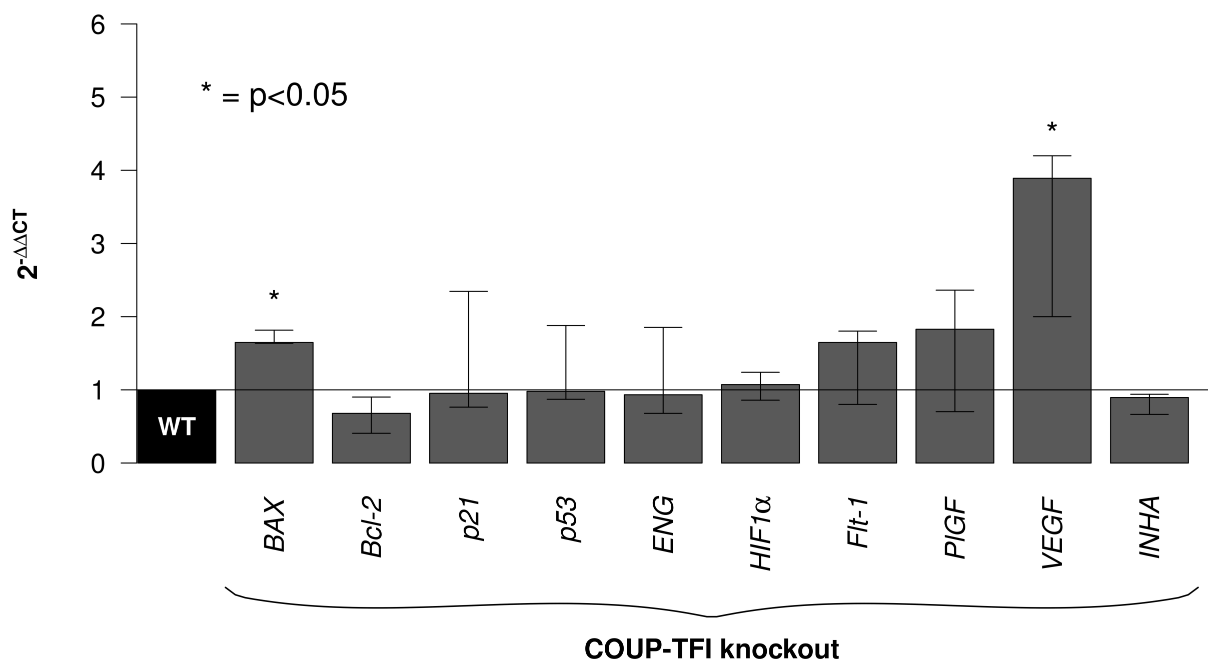

Fig. 1. Key placental genes expression pattern. Box plot showing fold change values ($2^{-\Delta\Delta Ct}$) of key placental genes in *COUP-TFI KO* (grey boxes) compared to *WT* (black box) levels. The values refer to the median and IQR and the p-values to the Wilcoxon test.

The performed reactions were run in triplicate in three independent experiments. The *mRNA* quantification was expressed in terms of the cycle threshold (Ct). From each triplicate run, the means of the Ct values were calculated and used for further analysis. All gene expression levels were normalized on the values of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Differences between the Ct values of the tested genes and those of the reference gene were calculated as ΔCt(gene) = Ct(gene) – Ct(*GAPDH*) and represented as $2^{(-\Delta Ct)}$ values. The relative fold changes in expression levels were determined as $2^{(-\Delta\Delta Ct)}$ that was determined by the following equation: $2^{(-(\Delta Ct(gene\ in\ KO)-\Delta Ct(gene\ in\ WT)))}$.

2.3 Statistical analysis

Data were analyzed using R v3.5.3 with $p < 0.05$ considered as significant. Based on preliminary data, a sample size of 4 mice per group, with α of 0.05 and 80% power, was found adequate to detect as significant a 1.5 fold difference in the expression of the targeted genes using a non-parametric test. To compensate for possible missing data, a total of 5 mice per group was considered. Distribution normality was tested by the Kolmogorov-Smirnov test. The T-test, Mann-Whitney-test, and Spearman test were performed as appropriate.

3. Results

According to RT-qPCR data, the most highly expressed gene in both *COUP-TFI KO* and *WT* placental tissue was *HIF1*α, followed by *Bax* and *p21*. In addition, *p21* resulted more expressed than *p53*, in both *COUP-TFI KO* and *WT* placental tissues (not shown). Interestingly, we found an increase of *VEGF-A* and *Bax* mRNA expression in placental tissue of *COUP-TFI KO* mice compared to their control counterparts. On the contrary, no significant differences were observed in mRNA expression of other marker of placental development, such as *Flt-1*, *PlGF*, *Bcl-2*, *p21*, *p53*, *VEGF-A*, *HIF1*α, *ENG*, and *INHA* (Fig. 1 and Table 2).

Table 2. Expression delta-CT values ($2^{(-\Delta CT)}$) of the studied mRNAs. The values are reported as median and IRQ and the *p*-values refer to the Wilcoxon test.

	COUP-TFI KO	*Wilde type (WT)*	*p*
BAX	0.035 (0.033–0.036)	0.020 (0.020–0.021)	<0.05
Bcl-2	0.003 (0.002–0.003)	0.003 (0.002–0.005)	0.841
p21	0.031 (0.023–0.047)	0.020 (0.017–0.051)	1.000
p53	0.001 (0.000–0.001)	0.000 (0.000–0.001)	0.690
ENG	0.004 (0.004–0.010)	0.008 (0.003–0.011)	1.000
HIF1α	0.047 (0.045–0.071)	0.061 (0.037–0.073)	1.000
Flt1	0.009 (0.007–0.018)	0.010 (0.005–0.010)	1.000
PlGF	0.015 (0.011–0.020)	0.008 (0.004–0.030)	0.548
VEGF1	0.010 (0.006–0.011)	0.003 (0.002–0.005)	<0.05
INHA	0.003 (0.001–0.003)	0.001 (0.001–0.003)	1.000

Correlations between all evaluated transcripts in *WT* and *COUP-TFI KO* mice are shown in Fig. 2. We found significant positive correlations in the placental tissue of

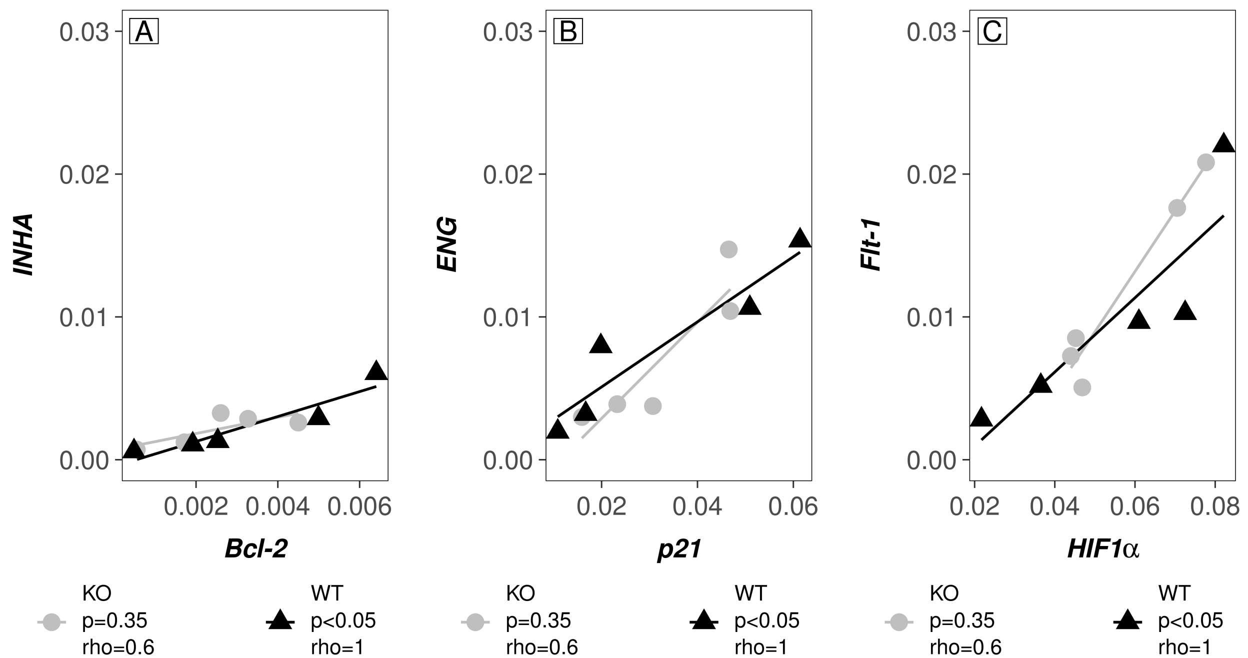

Fig. 2. Plots showing correlations between placental genes in *WT* and *COUP-TFI KO* mice. Panel (A) Shows correlation between *INHA* and *Bcl-2*. Panel (B) Shows correlation between ENG and p21. And Panel (C) Shows correlation between *HIF1α* and *Flt-1*. *p*-values and rho refer to Spearman test.

WT mice between the following mRNA pairs: *Bcl-2* and *INHA* (rho = 1 and $p < 0.05$); *p21* and *ENG* (rho = 1 and $p < 0.05$); *HIF1α* and *Flt-1* (rho = 1 and $p < 0.05$). Notably, such positive correlation between *Bcl-2* and *INHA*, *p21* and *ENG*, and between *HIF1α* and *Flt-1* mRNA expression was lost in mutant placentas. Additional positive correlations were found in WT placental tissue between the following pairs, even though they did not reach statistical significance (not shown): *p21* and *p53* (rho = 0.9 and p = 0.083); *p21* and *HIF1α* (rho = 0.9 and p = 0.083); *p21* and *Flt-1* (rho = 0.9 and p = 0.083); *p21* and *PlGF* (rho = 0.9 and p = 0.083); *ENG* and *p53* (rho = 0.9 and p = 0.083); *ENG* and *HIF1α* (rho = 0.9 and p = 0.083); *ENG* and *Flt-1* (rho = 0.9 and p = 0.083); and *ENG* and *PlGF* (rho = 0.9 and p = 0.083). All tested correlations were no longer significant in the placental tissue of *COUP-TFI KO* samples, where we only found a negative correlation between *Bcl-2* and *PlGF*, even though not significant (rho = –0.9 and p = 0.083).

Finally, we found a reduction of weight in *COUP-TFI KO* pups compared to littermates controls. The mean weight of WT mice was 1.6 grams (± 0.14), compared to 1.3 grams (± 0.13) of *COUP-TFI KO* mice ($p < 0.05$).

4. Discussion

In this study, we assessed the role of mouse *COUP-TFI* in regulating the expression of major genes involved in placentation. We found that *VEGF-A* and *Bax* mRNA expression were increased in *COUP-TFI KO* compared to wild-type mouse placentas, suggesting an impairment of apoptotic and angiogenetic pathways in mutant placental tissue. The positive correlations observed in normal placental tissue between *Bcl-2* and *INHA*, *p21* and *ENG*, and *HIF1α* and *Flt-1* were lost in *COUP-TFI* mutants, suggesting that key molecular networks could be imbalanced upon *COUP-TFI* loss. Interestingly, mutant mice also showed a significantly lower weight at birth compared to wild-type mice, raising the possibility that placental impairments described above could ultimately converge in sub-optimal weight gain during gestation.

We focused our attention on *COUP-TFI* because this family of nuclear receptors carries out vital roles in physiological processes, including proliferation, apoptosis and cell signaling [15,20]. Thanks to the level of evolutionary conservation of *COUP-TFI*, understanding the pathological pathway in relation to its expression in mouse models could help to better focus future studies on genes known to be relevant during the process of physiological placental development. In particular, we analyzed the expression of *HIF1α*, *ENG*, *Flt1*, *PlGF*, *VEGF-A*, which are main players in angiogenesis and vascular pathfinding, *p21* and *p53* in cellular proliferation, as well as *Bax*, *Bcl2* and *INHA*, which are involved in apoptosis and cell survival. All these genes regulate crucial aspects of placental development, and their variation was shown to be associated with impaired development of the ongoing pregnancy [21,22].

When considering the influence of *COUP-TFI* on the expression of the most common angiogenetic factors, we found that *VEGF-A* mRNA was consistently up-regulated

in *COUP-TFI KO* placentas compared to control mice. The isoform A of the vascular endothelial growth factor (*VEGF-A*), belonging to the VEGF family, is considered the most crucial factor promoting the differentiation of mesenchymal cells in villi into hemangioblastic stem cells. *VEGF-A* expression is induced by hypoxia, as a potent stimulus, and is mediated via *HIF1*α expression [23–26]. Indeed, *VEGF-A* is strongly expressed by the cytotrophoblast cells during the first trimester of pregnancy and strong evidence indicates that high *VEGF-A* expression in fetal growth restriction reflects the hypoxic status of the placenta [27,28].

Supporting this evidence, we found in *COUP-TFI* mutants a loss in the positive correlation between *mRNA* expression of *HIF1*α and *Flt-1*, this latter encoding the vascular endothelial growth factor receptor 1 (*VEGFR1*), one of the receptors for vascular endothelial growth factors (*VEGF*). In a hypoxic environment, *Hif1*α could regulate the expression of *VEGF-A*, *Flt-1* and other angiogenic factors, by means of a compensatory mechanism aimed to restore normal placental blood flow and to rescue normoxia [29,30]. This finding overlaps with studies showing that *VEGF-A mRNA* and protein levels are significantly reduced in patients with growth restriction and that an adenovirus-mediated overexpression of *VEGF* can improve fetal growth in a sheep model [31,32]. Regarding the other angiogenic factors considered in our study, *PlGF* and *ENG*, no significant differences were found in *COUP-TFI KO* placentas compared to control mice.

Considering the most common genes involved in cell proliferation and survival control, we observed an increase of *Bax* mRNA in mutant placentas. An augmented *Bax* expression is in line with other studies conducted on human placenta [33–35]. *Bax* is a pro-apoptotic protein that exerts, in concert with the anti-apoptotic protein *Bcl-2*, a crucial role in apoptosis. Both are regulated by the *p53* tumor suppressor gene [36]. Apoptosis contributes to the turnover of villous trophoblasts and plays a crucial function in the remodeling of spiral arteries in human placenta. Apoptosis in placental villi changes throughout normal pregnancy: it is low in the first trimester, increases in the second, and then reaches the highest levels beyond 40 weeks of gestation [37]. Furthermore, the amount of apoptosis is increased in villous trophoblast in placental pathologies, including preeclampsia [38].

In addition, we observed a significant positive correlation of *Bcl-2* and *INHA* in normal mice. This could be due to a regulatory role of on trophoblast growth through inhibition of the activin receptor, known to have a fundamental role in trophoblast development and correct placentation [39]. This, in turn, could result in reduced placenta proliferation and increased apoptosis characterizing old placentas at the end of gestation [40]. According to our study, this correlation seems to be altered by the absence of *COUP-TFI*.

Interestingly, we also observed a lower level of *p53* expression than its downstream target *p21* in *COUP-TFI KO* samples, and a positive correlation of the expression rate of the two genes. These data confirmed previous results on human placentas [21]. We can further hypothesize a role of *p21* independent of *p53*. Usually *p53*, through *p21*, promotes cell cycle arrest or apoptosis *via* the augmented expression of *Bax* [36,41]. In the current study, we found a significant positive correlation between *p21* and *ENG* transcripts. *ENG* is part of the transforming growth factor-beta receptor complex. Angiogenesis, apoptosis, and cell cycle arrest could be promoted by the transforming growth factor-beta pathway, reported to be implicated in fetal growth restriction [42]. *p21* could also possibly interact in placental tissue with this cascade triggered by the transforming growth factor-beta receptor complex [43]. As the correlation between *p53* and *p21* is lost in mutant mice, this interaction could be disturbed by the absence of *COUP-TFI*.

Finally, we looked at the link of the mouse phenotype to placental function in terms of weight of pups recorded at birth. Interestingly, our results showed that *COUP-TFI KO* pups presented a significant lower weight than WT littermate controls. These data further support the pathological significance of *COUP-TFI* in placental development, potentially related to fetal growth restriction, a common complication associated with impaired placental function in humans [9,44,45]. Our preliminary results invite further studies on specific downstream cascades of molecular markers linked to *COUP-TFI*, both in mouse models and humans.

This explorative study aimed to identify potential markers involved in impaired placental function linked to *COUP-TFI* loss-of-function but lacks a detailed analysis of mechanisms underlying the downstream regulation of angiogenic, cell regulation and apoptotic factors included in this mouse model. Moreover, in our experiments we have not assessed the function COUP transcription factor 2 (*COUP-TFII*), a homolog to *COUP-TFI* sometimes compensating *COUP-TFI* functions. *COUP-TFI* and *COUP-TFII* expression patterns overlaps in many regions and organs, possibly resulting in redundant functions [6,46,47]. Thus, *COUP-TFII* may be able to compensate for the absence of *COUP-TFI* in *COUP-TFI KO* mice. Further experiments on placentas lacking both COUP-TF members derive from crossing between *COUP-TFI* and *COUP-TFII* mutant mice, could shed new light on the interplay between these nuclear receptors during placental development. Exploring in more detail placental morphology could also be a topic of future interest.

5. Conclusions

The present study provides evidence that the absence of *COUP-TFI* influences the expression levels of two key effectors of mouse placental angiogenesis and apoptosis, *VEGF-A* and *Bax*. Consistently, we showed that *COUP-TFI KO* mice presented a significant lower weight at delivery than WT littermate controls. Hence, we propose

that *COUP-TFI* plays an important role for placental development and function, even though further studies will be necessary to dissect the molecular dynamics governing a *COUP-TFI*-dependent placenta development.

Abbreviations

Bax, BCL2-associated X protein; *Bcl-2*, B-cell lymphoma 2; *COUP-TFI*, Chicken Ovalbumin Upstream Promoter-Transcription Factor I; *COUP-TFII*, Chicken Ovalbumin Upstream Promoter-Transcription Factor II; *ENG*, Endoglin; *FLT-1*, Fms-like tyrosine kinase 1 (also known as VEGFR1); *HIF1α*, Hypoxia inducible factor 1 alpha subunit; *INHA*, Inhibin alpha; mRNA, Messenger ribonucleic acid; *VEGF-A*: Vascular Endothelial Growth Factor A.

Author contributions

LV, SM, APL, MS, LM, AF—substantial contributions to conception and design. LV, SM, MB, APL, MO, SB, MS, LM, AF—substantial contributions to acquisition of data or to analysis and interpretation of data. LV, SM, MB, APL, MO, SB, LD, CDL, MS, LM, AF—drafting the article or revising it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All mouse experiments were conducted in accordance with the relevant national and international guidelines and regulations (European Union rules; 2010/63/UE), and with approval by the local ethical committee in France (CIEPAL NCE/2019-548).

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Conflict of interest

The authors declare no conflict of interest. APL is the Editor of this journal, given his role as Editor, had no involvement in the peer-review of this article and has no access to information regarding its peer-review.

Availability of data and material

The data that support the findings of this study are available, but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of the Internal Review Board.

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