COUP-TFI Deletion Affects Angiogenesis and Apoptosis Related Genes Expression in Ex-Vivo Mice Placenta

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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. Aim of the study was to investigate whether the absence of this gene affects placental development and fetal growth in a mouse model. For this scope placentas of COUP-TFI-knockout (COUP-TFI –/–) and wild-type (WT) were collected at the 18.5 days post-coitum. mRNA amount of following genes known to be involved in different key molecular pathways were evaluated: Bax and Bcl-2 (apoptosis), p21 and p53 (proliferation), VEGF-A, PlGF, HIF1α, Flt-1, ENG, and INHA (angiogenesis). Mice litter weight at birth was also compared.

Results: RT-qPCR analysis showed an increased mRNA expression of VEGF-A and Bax in placental tissue of COUP-TFI –/– mice compared to WT mice. Also, a lost in the positive correlation between Bcl-2 and INHA, p21 and ENG, as well as HIF1α and Flt-1 mRNA expression in COUP-TFI null mutants was found. Finally, the mean weight of WT mice was 1.6 g (±0.14) compared to 1.3 grams (±0.13) of COUP-TFI –/– mice ($p<0.05$).

Conclusions: Our results show that COUP-TFI deletion is associated to a lower birth weight in mouse and an increased placental mRNA expression of pro-apoptotic Bax and pro-angiogenetic VEGF-A genes.

Introduction

At the base 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 trophoblast, further develop into the maternal decidua and give 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, included a deficient growth of ongoing pregnancy (2).

Compared to the monolayer human placenta (hemomonochorial), **placenta in mice** is composed by three trophoblast layers (hemotrichorial) (3). As at day E10.5 the mid-gestation phase begins, all the layers of the placenta are constituted, including the outermost maternal part, called the decidua, and the fetal part with the triple trophoblastic layer (4,5). Embryonic development ends with the mid-gestation phase at day E13.5, thereafter the fetus matures until the time of birth, around day E19.5 (6).

COUP-TFI (Chicken Ovalbumin Upstream Promoter-Transcription Factor I), also known as NR2F1 (Nuclear Receptor subfamily 2, group F, member 1) is an orphan nuclear receptor factor 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 (7,8). Overall,

the high conservation of amino acid sequence between species suggests vital evolutionarily conserved functions worth to be explored (9–11).

Previous studies conducted in human placenta highlight the potential key role of the transcription factor COUP-TFI. An in-silico investigation of transcriptional profile obtained using a microarray approach, revealed that COUP-TFI was highly associated with self-renewal and differentiation of human chorionic trophoblast progenitor cells (12). Also, 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 (13).

Engineered COUP-TFI -/- mice have been developed to share more light into COUP-TFI mechanisms of function of this gene. In this condition, 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 (10). However, it has not yet been evaluated if and how COUP-TFI could influence the process of placental development.

Aim of this study was to explore 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.

Materials And Methods

Animals

COUP-TFI null (COUP-TFI −/−) mice, as previously described, were generated and subsequently genotyped using the following primers: forward 5'-CTGCTGTAGGAATCCTGTCTC-3', reverse 5'-AATCCTCCTCGGTGAGAGTGG-3' and reverse 5'-ACATACACAGCCTGGCCTTGC-3' (14–16). Heterozygous mice were bred together to generate COUP-TFI −/− 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 ethic committee (Comité Institutionnel d'Éthique Pour l'Animal de Laboratoire, CIEPAL, NCE/2011-23) (17). One pregnant female with embryonic mice at a time was euthanized and placentas collected after terminal dissection. In the study 10 placentas were analyzed. Five of them were wild type and 5 were mutant (COUP-TFI −/−).

Real-time PCR

All samples were transferred to 500 µl of TRIzol Reagent (Invitrogen) and processed for total RNA isolation according to the manufacturers' protocol. RNA (1 µg) was reverse transcribed by use of the SuperScript® III REV trascript (Life Technologies). The quantitative RT-PCR reaction was performed using

Roche LightCycler® 480 and SSOADV UNIVER SYBR GRN SMX 500 (BIO-RAD), according to the manufacturers' protocols.

The following genes have been included as markers for placental development: Hif1α, ENG, Flt1, PlGF and the isoform A of the VEGF (VEGF-A), being among the main players involved in angiogenesis pathway, P21 and P53 for cell proliferation and Bax, Bcl2, and 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'	HIF1A
Antisense 5'-GGAATGGGTTCACAAATCAGCAC – 3'	HIF1A
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

Table 2
Expression delta-CT values ($2^{(-\Delta CT)}$) of the studied proteins.

	COUP-TFI−/−	Wilde type	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
HIF1A	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

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 in the further analysis. All the assays were normalized regarding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values. Differences between the Ct values of the tested genes and those of the reference genes were calculated as ΔCt(gene) = Ct(gene)−Ct(GAPDH) and represented as 2(-ΔCt) values. The relative fold changes in expression levels were determined as 2(-ΔΔCt) that was determined by the following equation: 2(-(ΔCt(gene in KO)-ΔCt(gene in WT))).

Statistical analysis

Data was analyzed using R v3.5.3 with $p < 0.05$ considered significant. Distribution normality was tested by the Kolmogorov-Smirfoff test. The T-test, Mann-Whitney-test, and Spearman test were performed as appropriate.

Results

According to RT-PCR, the most expressed genes were HIF1α, followed by Bax and p21 both in COUP-TFI −/− and WT placental tissue. In addition, p21 resulted more expressed than p53 both in COUP-TFI −/− and WT placental tissue (not shown). Compared to WT mice, our study shows an increase of VEGF-A and Bax mRNA expression in placental tissue of COUP-TFI −/− mice. No significant differences were observed in mRNA expression for Flt-1, PlGF, Bcl-2, p21, p53, VEGF-A, HIF1α, ENG, and INHA (Fig. 1).

In Fig. 2 we show the correlations between all studied transcripts in WT and COUP-TFI −/− mice. In the placental tissue of WT mice we found significant positive correlations 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 $p < 0.05$). This positive correlation between Bcl-2 and INHA, p21 and ENG, and between HIF1α and Flt-1 mRNA expression was found to be lost in the COUP-TFI null mutants. Besides these results, we found other positive correlations in placental tissue of WT mice between the following pairs, even though not significant (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 −/− mice. There was only a negative correlation between Bcl-2 and PlGF, even though not significant (rho=-0.9 and $p = 0.083$).

Finally, considering the weight of mouse litter, this was found to be reduced in COUP −/− mice. The mean weight of WT mice was 1.6 grams (± 0.14) compared to 1.3 grams (± 0.13) of COUP-TFI −/− mice ($p < 0.05$).

Discussion

This was a mouse model study conducted to assess the role of the COUP-TFI gene in the expression pattern of some major genes involved in the process of placentation. Here we found that VEGF-A and Bax mRNA expression was augmented in COUP-TFI null mice compared to wild-type mice placentas. The positive correlations between the following mRNA pairs observed in normal placental tissue were lost in COUP-TFI null mutants: Bcl-2 and INHA; p21 and ENG; HIF1α and Flt-1. Interestingly, COUP-TFI null mice resulted also to have a significantly lower weight at birth compared to wild-type mice.

We focused our attention on COUP-TFI because this family of proteins carries out vital roles in physiological processes, including proliferation, apoptosis and cell signaling (18). Thanks to the level of conservation of COUP-TFI, understanding the pathological pathway in relation to its expression in mice models could help to focus future studies on genes that are 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 (19,20).

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 −/− 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 HIF1α expression (21–24). 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 (25,26).

Supporting this evidence, we found in COUP-TFI null mutants a lost in the positive correlation between mRNA expression of **HIF1α** 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, Hif1α could regulate the expression of VEGF-A, Flt-1 and other angiogenic factors, being this a compensatory mechanism aimed to restore normal placental blood flow and rescue the normoxia (27,28). This finding overlap 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 (29,30). **Regarding the other angiogenic factors** considered in our study, **PlGF and ENG**, no significant differences were found in COUP-TFI −/− 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 COUP-TFI−/− placentas. An augmented Bax expression is in line with other studies conducted on human placenta (31–33). 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 (34). 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 (35). Furthermore, the amount of apoptosis is increased in villous trophoblast in placental pathologies, including preeclampsia (36).

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 INHA on trophoblast growth through inhibition of activin receptor, known to have a fundamental role in trophoblast development and correct placentation (37). This, in turn, could result in reduced placenta proliferation and increased apoptosis characterizing old placentas at the end of gestation (38). According to our study, this correlation seems to be disturbed by the absence of COUP-TFI.

Interestingly, we also observed in COUP-TFI −/− mice a level of p53 expression that was lower than its downstream target **p21** and that the expression rate of the two genes positively correlated. This data confirmed previous data on human placentas (20). We could further hypothesize a role of p21 independent from p53. Usually p53, through p21, promotes cell cycle arrest or apoptosis through the augmented expression of Bax (34,39). In the current study, we found a significant positive correlation between p21 and ENG. 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 (40). p21 could possibly interact in placental tissue with this cascade triggered by transforming growth factor-beta receptor complex (41). As the

correlation between p53 and p21 is lost in COUP-TFI -/- mice, this interaction could be disturbed by the absence of COUP-TFI

Finally, we looked at mice phenotype linker to placental function in terms of weight of pups recorded at birth. Interestingly, our results showed that COUP-TFI −/− mice presented a significant lower weight than WT 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 (13,42,43). Our preliminary results support further studies on specific downstream cascades of molecular markers found to be linked to COUP-TFI, both in mouse models and humans.

Limits of the study

This was an explorative study performed to find out potential markers involved in impaired placental function linked to COUP-TFI disruption. Thus, this study lacks a detailed study 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-TFII, a highly redundant to COUP-TFI transcription factor. COUP-TFII, being more expressed in developing organs, shows a high degree of homology at the amino acid level with COUP-TFI. Even though above-mentioned peculiarity, COUP-TFI and COUP-TFII expression patterns overlaps in many areas, possibly resulting in redundant functions (9,44,45). Thus, COUP-TFII may be able to mitigate for the absence of COUP-TFI in COUP-TFI −/− mice. Further experiments on placentas lacking both COUP-TFI and COUP-TFII deriving from matings between COUP-TFI −/− and COUP-TFII −/− mutant mice, could shed new light on the interplay between these two orphan receptors during placenta development.

Conclusions

The present study provides ex-vivo evidence that the ablation of COUP-TFI influences two effectors of placental angiogenesis and apoptosis, VEGF-A and Bax, in the murine placenta. Furthermore, we showed that COUP-TFI −/− mice presented a significant lower weight at delivery than WT controls. Thus, even though molecular dynamics during placenta development remain still a large territory to explore, it is possible to speculate a role of COUP-TFI expression in placental function.

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.

Declarations

Ethics approval and consent to participate

The study was approved by the local ethic committee (Comité Institutionnel d'Éthique Pour l'Animal de Laboratoire, CIEPAL, NCE/2011-23).

Consent for publication

Not applicable.

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 was 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.

Competing interests

The authors declare that they have no potential conflicts of interest relevant to this article.

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Contribution to Authorship

Substantial contributions to conception and design or 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 (LV, SM, MB, APL, MO, SB, LD, CDL, MS, LM, AF). All authors read and approved the final manuscript.

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Figures

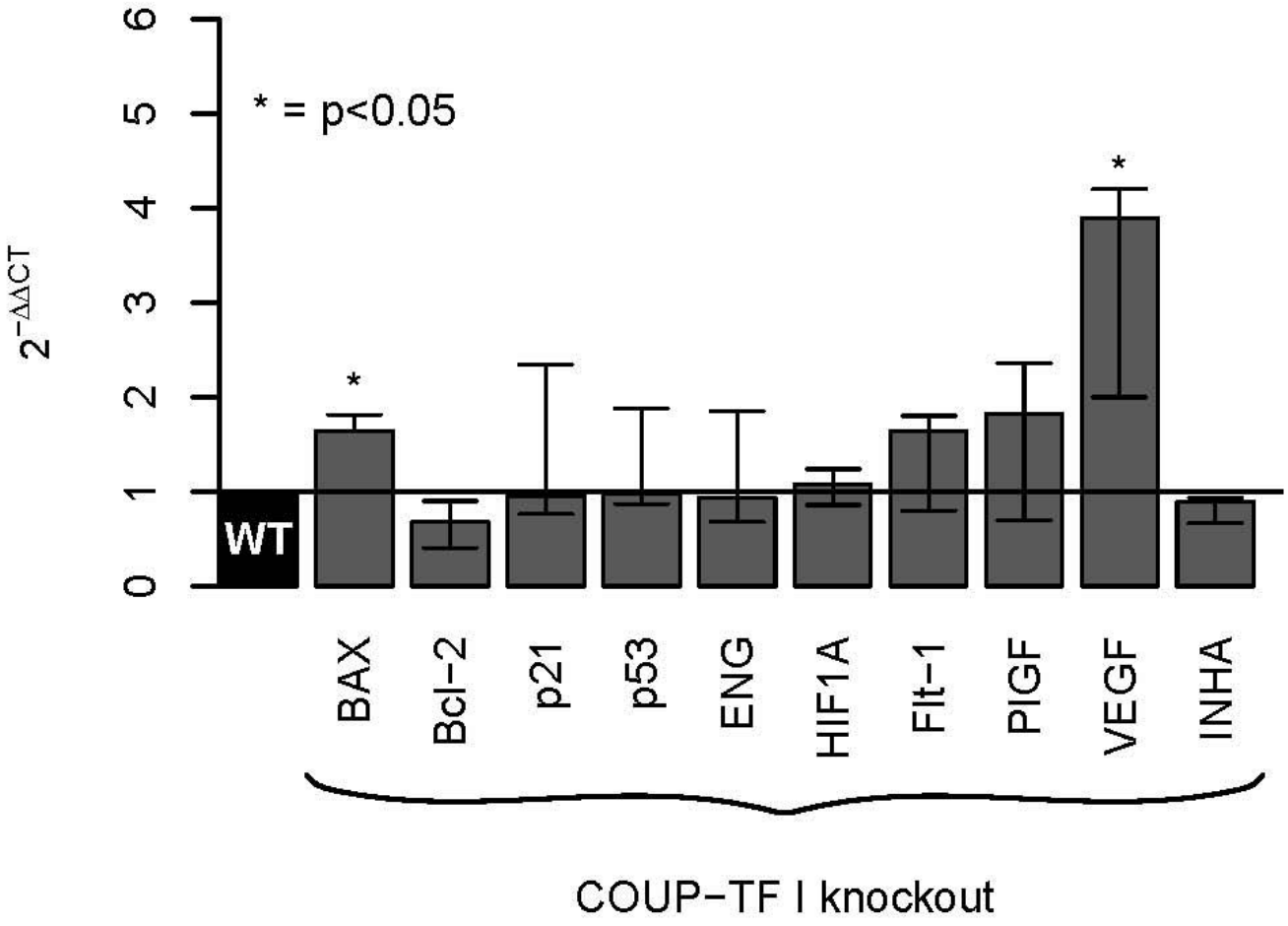

Figure 1

Plot showing fold change (2−ΔΔCt) of studied mRNAs in placental tissue of COUP-TFI−/− in comparison to placental tissue of WT mouse.

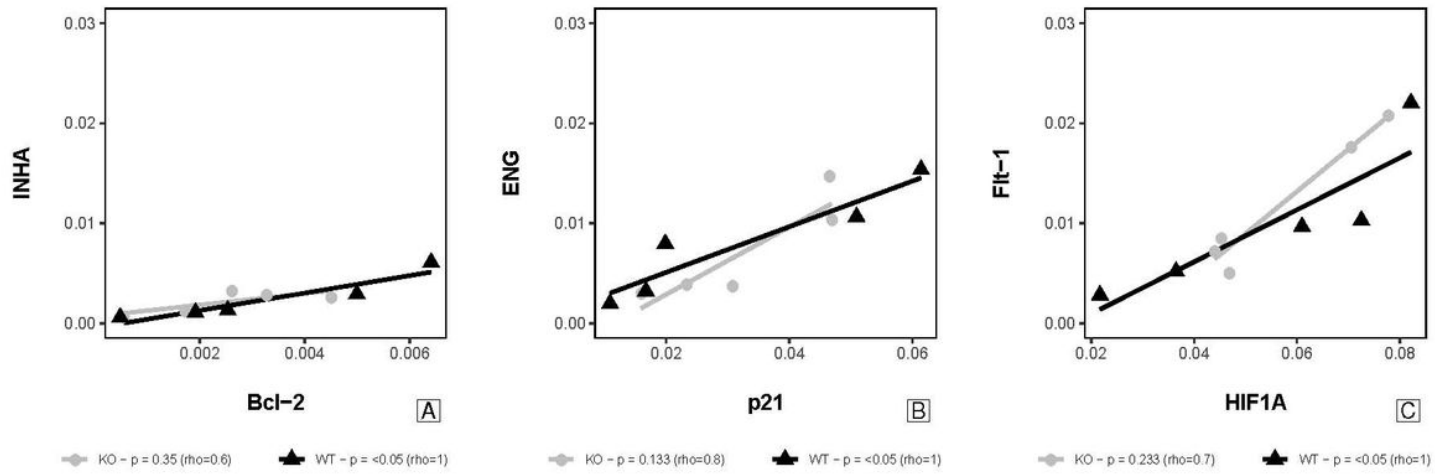

Figure 2

Plots showing correlations: in WT mice and in COUP-TFI−/− mice. P-values and rho refers to Sperman test.

Supplementary Files

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