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Untangling the placentome gene network of beef heifers in early gestation

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Untangling the placentome gene network of beef heifers in early gestation

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ABSTRACT

The cotyledon and caruncle tissues provide a functional bridge between the fetus and the dam. However, the relationship between these tissues and the transcriptomic profile that underlies the tissue functions remains elusive. Herein we investigate the expression profile of cotyledon and caruncle from nulliparous beef heifers carrying female fetuses at day 83 of pregnancy to identify changes occurring across tissues that contribute to placental function and their tissue-specific roles. We identified 2654 differentially expressed genes [$padj \leq 0.05$, abs(log2FC) ≥ 1], including nutrient transporters and paternally imprinted genes. We found key regulators of tissue function and differentiation, including *FOXO4*, *GATA2*, *GATA3*, and *HAND1,* rewired between the tissues. Finally, we shed light on the over-represented pathways related to immune tolerance, tissue differentiation and remodeling. Our findings highlighted the intricate and coordinated cross-talk between fetal-maternal tissues. They provided evidence of a fine-tuned gene regulatory network underlying pregnancy and tissue-specific function in the bovine placenta.

1. Introduction

The placenta is formed by an intricate and tightly regulated relationship between fetal and maternal tissues that work together to ensure a successful pregnancy [1]. In so-called cotyledonary placentas (giraffes, pronghorn antelope, cattle and other bovids including waterbuck, gnu, impala, goats, and sheep), the fetal cotyledons (COT) and maternal caruncles (CAR) form a placentome, which provides a functional bridge between the fetus and the mother [1–3]. This differs from other placental types, such as hemochorial placentas of primates, rodents and bats, or the endotheliochorial placenta of carnivores, in which the maternal endometrial contribution to the placenta is partially eroded [1]. Thus, the cotyledonary placenta is ideally suited to study the fetal (COT) vs. the maternal (CAR) portions of the placenta.

The complexity of mammalian placental function goes beyond its role in transporting nutrients and metabolic wastes. For example, the placenta plays a pivotal role in implantation, maternal recognition of pregnancy through hormonal and growth factor synthesis, and immunomodulatory responses [4–6]. In addition, the physiological, biochemical, and molecular processes in conceptus implantation and placentation are spatially and temporally regulated to sustain fetal development [6,7]. The first half of gestation is marked by increased placental growth, whereas placental function increases throughout pregnancy (~ 280 days in cows) to provide nutrients to the growing fetus [8]. These synchronous processes are coordinated through molecular and physiological pregnancy-related mechanisms, including fetal-maternal interactions and a genomic and epigenomic multilayered regulatory network [3,6,9,10]. Despite the growing knowledge on placentation and the fetal-maternal relationship, the genomic basis underlying this cross-talk has received little attention.

A growing number of studies have reported the placental transcriptional and regulatory profiles of humans and animal models [4,11,12].

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Saben et al. [4] described the human placenta transcriptome, and Gong et al. [11] provided a deep characterization of coding and non-coding RNAs underlying placental function in health and disease. At the same time, most of the studies in livestock species have focused on a few candidate genes [12–14]. To the best of our knowledge, there is no genome-wide transcriptomic study in cattle that describes the differences between the fetal and maternal placental profiles. Thus, our understanding of the complexity of the COT/CAR relationship in livestock is still rudimentary. Lotfan et al. [13] reported 1075 DEGs by comparing the expression of pregnant fetal COT with non-pregnant CAR in buffaloes. Likewise, Murugesan et al. [14] profiled coding and long non-coding RNAs of CAR from pregnant and non-pregnant buffaloes.

Despite increased knowledge of the fetal-maternal relationship leading to a fine-tuning of gene expression, the genetic interplay between COT and CAR tissues of beef heifers in early pregnancy is still to be unveiled. Furthermore, it remains elusive to untangle the biological

Fig. 1. Transcriptomic profiles of cotyledon (COT) and caruncle (CAR) tissues from beef heifers at day 83 of pregnancy. (**A**) Differentially expressed genes (DEGs) coding nutrient transporters. Substrates were compiled from [15,20,21]. (**B**) Key transcription factor (TFs) expression profiles identified by RIF. Genes in bold were found as both DEGs and key TFs. Up and downregulation are color-coded as presented in the legend.

processes and pathways underlying the specific changes in the fetal and maternal tissues during placentation. Thus, we designed this study to investigate the transcriptomic profile of COT and CAR tissues and identify the changes occurring across tissues that contribute to placentation and tissue-specific functions.

Herein we report a comprehensive and systematic analysis of COT and CAR transcriptomic profiles from beef heifers (primiparous) at the end of the first trimester of pregnancy (day 83 $\pm$ 2). We first identified up and downregulated genes by comparing the expression profiles of the fetal (COT) vs. the maternal (CAR) portions of the placenta. Furthermore, we reported differentially expressed nutrient transporters and imprinted coding genes. Next, we inferred key regulators (transcription factors, TF) of tissue-specific function and differentiation. We created regulatory networks to model the gene-gene relationships and tissue-specific gene rewiring through co-expression and clustering analyses. Finally, we found that genes underlying pathways, such as PI3K-AKT, MAPK, and Wnt, had different expression profiles between the fetal and maternal placental tissues.

2. Results

We applied a comprehensive and systematic analysis on CAR and COT transcriptomic profiles of beef heifers at day 83 of pregnancy. The genome-wide RNA-Seq of seven paired COT and CAR samples generated a total of 321.2 M reads ($\sim$ 22.9 M reads per sample) (Table S1 – Table 1). Mapping reads to the ARS-UCD 1.2 reference genome revealed that, on average, 97% of reads were uniquely mapped. Based on an integrative approach, herein, we reported significant differentially expressed genes (DEGs) and co-expressed networks of genes that underlie COT and CAR function. Furthermore, we retrieved nutrient transporters, imprinted genes, and key TFs modulating gene expression, tissue regulation, and function.

2.1. Differential expression of COT vs. CAR tissues

From stringent quality control, 14,312 genes were tested for differential expression out of 27,607 reported on the *Ensembl* annotation file. We followed the DESeq2 approach to identify DEGs by contrasting COT vs. CAR [*Padj* value cut-off $\leq$0.05 and abs(log2FC) $\geq$ 1]. Among the 2654 DEGs, 63% were downregulated in COT. While most of the DEGs were protein-coding, we also identified long ($n = 62$) and small ($n = 22$) non-coding RNAs, including bta-miR-431 and miR-433, that were upregulated in COT (Table S1 – Table 2).

To understand the maternal-fetal cross-talk that underlies nutrient transport, we screened the DEGs list to identify nutrient transporter-coding genes [15]. We found 78 genes involved with the fetal provision of nutrients, mainly from the ABC and SLC nutrient transporter families (Fig. 1A). Interestingly, while most of the genes involved with amino acid transport were upregulated in COT, those providing lipids were downregulated.

2.2. Placental imprinted genes and key transcription factor regulators (TFs)

To shed light on the putative functional roles of the placental transcripts that were differentially expressed, we searched for imprinted genes that were included in the DEG list. Based on the imprinted gene database [16], we identified 14 and 36 DEGs that were previously reported as imprinted in cattle or humans, respectively (Table S1 – Table 3.) Interestingly, 10 DEGs were reported as paternally imprinted in cattle, and eight of them were upregulated in COT. Among the cattle imprinted genes found here, we can highlight *DIO3, IGF2, MEST, PEG3, PEG10, PLAGL1,* and *DIRAS3*. Except for *DIRAS3*, all the other imprinted genes were upregulated in COT. By overlapping the DEGs with the human imprinted gene list, we found that *GATA3* is paternally imprinted and was upregulated in COT. Conversely, *HOXA2* and *HOXA11* were downregulated and maternally imprinted.

Based on the regulatory impact factor metric (RIF) [17], we prioritized key regulator TFs potentially modulating the expression of differentially expressed genes between COT and CAR and driving tissue-specific functions. Using RIF1 and RIF2 to test 863 TFs from the Animal TF database v3.0 [18] and expressed in the placentome (COT and CAR portions of the placenta), we identified 84 key TFs (Fig. 1B) grouped into 79 families (*p-value* $\leq$ 0.05). Among these 79 families, the zinc finger C2H2 and homeobox were over-represented by 18 and 9 TFs, respectively (Table S1 – Table 4). From RIF1, the TFs *AFF2* (*z-score* = $-$3.804) and *NRF2* (*z-score* = $-$ 3.636) showed the most extreme negative values, whereas *ARID3A* (*z-score* = 3.057) and *ZFP57* (*z-score* = 2.965) showed the greatest positive values. Likewise, for RIF2, we found *FOXO4* (*z-score* = $-$ 3.038) and *GATA2* (*z-score* = $-$ 2.944) as the extreme negative and *ZNF391* (*z-score* = 2.367) and *GLMP* (*z-score* = 2.502) as the extreme positive. Interestingly, among the key TFs, we identified 33 that were DEGs. Most of these differentially expressed TFs were upregulated in COT, including *ARID3A, FOXO4, GATA2, GATA3, HAND1, PPARD,* and *PPARG*. Conversely, among the downregulated TFs were *HOXD10, HOXB2, THRB*, and *ZNF711*. The significant TFs identified as RIF1 and RIF2 are reported in Table S1 – Table 4.

We performed a functional over-representation of biological processes (BP) and KEGG pathways to understand the biological role of DEGs and key TFs in the bovine placentome. First, we broke down the BP, which were differentially regulated based on the tissue, by analyzing up and downregulated genes separately (Table S1 – Tables 5 and 6). This approach retrieved embryonic, tissue, and animal organ morphogenesis among the over-represented BP that underlie the upregulated genes in COT (Fig. 2A). Additionally, and not surprisingly, the downregulated genes were acting mainly in immune system-related processes such as cytokine production, leukocyte migration, and inflammatory response (Fig. 2B). Second, we analyzed DEGs and key TFs under a cluster analysis framework implemented in ClueGO [19] to identify KEGG pathways. Based on this analysis, we identified ten over-represented KEGG pathways (Fig. 2C) that included ovarian steroidogenesis, thyroid hormone synthesis, glutathione metabolism, and ECM-receptor interaction (FDR $\leq$ 0.05). The list of pathways and underlying genes for all clusters is reported in Table S1 – Table 7.

2.3. Transcription factors are hubs in COT and CAR co-expression networks

We further explored the functional relationship between gene pairs within placental tissues to investigate the specificities of gene rewiring and the underlying biological processes responsible for the differences in CAR and COT regulation. First, using the partial correlation and information theory (PCIT) algorithm [22], we constructed tissue-specific networks using the co-expression patterns of 2705 genes that included the DEGs and key TFs. Second, we used gene connectivity to determine tissue-specific gene rewiring by applying the concept of differential connectivity (DK) [23]. Lastly, we used the *k-means* approach to cluster genes with similar expression behavior across COT and CAR and shed light on the biological processes.

Our network analysis retrieved 220,273 and 228,578 significantly co-expressed pairs from CAR and COT, respectively ($p \leq 0.05$). To reduce the data dimensionality and narrow down the biological relationships, we kept only gene pairs with a $|r| \geq 0.8$. Thus, 6660 pairs (1431 unique genes) for CAR (Table S2 – Table 1) and 2829 pairs for COT (1620 unique genes) were kept for further analysis (Table S2 – Table 2). Then, we used connectivity as a measure of centrality to select genes with a high degree (hub genes). The CAR tissue showed greater average connectivity (14.40) than COT (11.33), and TFs presented the greatest connectivity in both tissues. We identified 61 and 59 hub genes from CAR and COT, respectively ($p \leq 0.05$). The *HAND1, PPARD,* and *FOXO4* showed the greatest connectivity within CAR, whereas *ZNF391, SATB2,* and *ENSBTAG00000038635* were highlighted in COT (Figs. 3

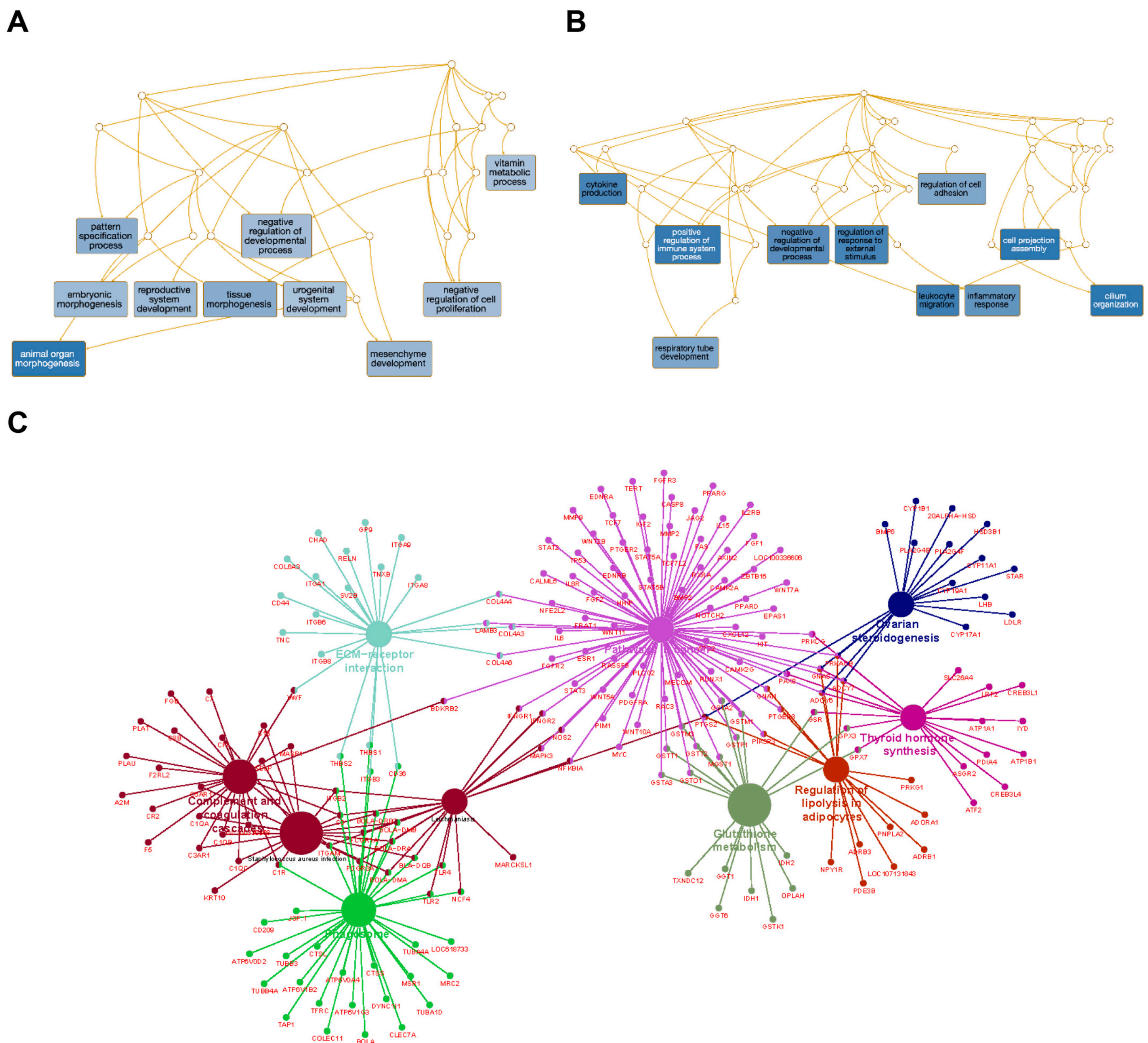

Fig. 2. Functional over-representation of differentially expressed genes and key transcription factors from placentomes of beef heifers at day 83 of pregnancy. Over-represented biological processes of upregulated (**A**) and downregulated (**B**) genes in cotyledon. (**C**) KEGG pathways of DEGs and transcription factors based on the cluster analysis framework from ClueGO. Significant terms were taken when FDR $\leq$ 0.05. Clusters are color-coded as follows: ECM-receptor interaction – cyan; pathways in cancer – magenta; ovarian steroidogenesis – dark blue; thyroid hormone synthesis – dark purple; regulation of lipolysis in adipocytes; glutathione metabolism – green; phagosome - fluorescent green; complement and coagulation cascades – dark red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and 4).

To identify the most rewired (differentially connected) genes between CAR and COT, we tested 2636 genes that were retrieved from the PCIT analysis ($|r| \geq 0.8$, $p < 0.05$). Based on that, we found 56 differentially connected genes (Table S2 – Table 3) ($p \leq 0.05$), and among them, 26 were differentially expressed as well. Among the *DK* genes, we identified 33 genes with a gain of connectivity in CAR, including *HAND1, PPARD, FOXO4, ZFP57,* and *TFP2A*. On the other hand, genes with increased connectivity in COT included *SATB2, NFATC2, NR2F2,* and *TCF12*.

We used *k-means* hierarchical clustering to identify functionally relevant gene groups from the CAR and COT gene sets. We clustered 4000 genes out of 14,312 with an SD $\geq$ 0.4 that were gathered in four clusters (Fig. 5A). Cluster A grouped 1747 genes that were highly expressed in COT. The remaining genes, however, were more expressed in CAR, mainly in clusters C (n = 1362) and D (n = 405). The genes gathered in each cluster and the *k-means* values are reported in Table S2 – Table 4. Supporting our hypothesis that specific pathways underlie the tissue-specific function and maternal-fetal cross-talk, we found different KEGG pathways over-represented in each cluster (Fig. 5B). Over-represented pathways from highly expressed genes in COT (cluster A) included key signaling pathways such as Wnt, PI3K-Akt, and MAPK. Furthermore, we found metabolic pathways involved with carbon metabolism, biosynthesis of amino acids, and steroid biosynthesis. From the remaining clusters, we can highlight pathways related to cell structure and remodeling, such as focal adhesion and ECM-receptor

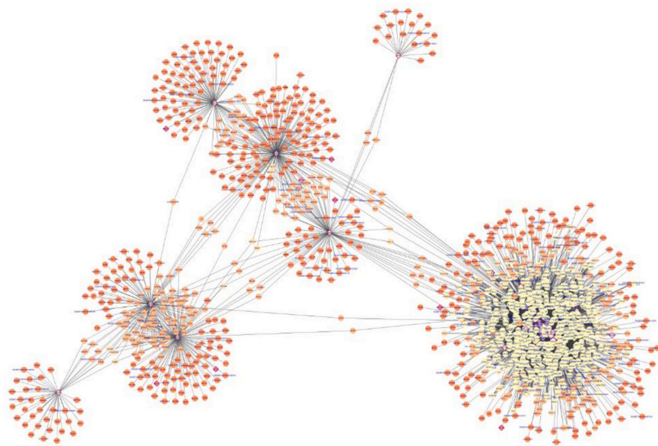

Fig. 3. Caruncle regulatory network of differentially expressed genes and key transcriptions factors of beef heifers at day 83 of gestation. Nodes are differentially expressed genes or key transcription factors with a $|r| \geq 0.8$. Nodes with a magenta border were differentially connected between cotyledon and caruncle. The node size and color (from light to dark) are proportional to the number of connections for each gene. Nodes with few connections not linked to the main network are not showed. Transcription factors are represented by a diamond shape. High-resolution image is provided in supplementary material. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

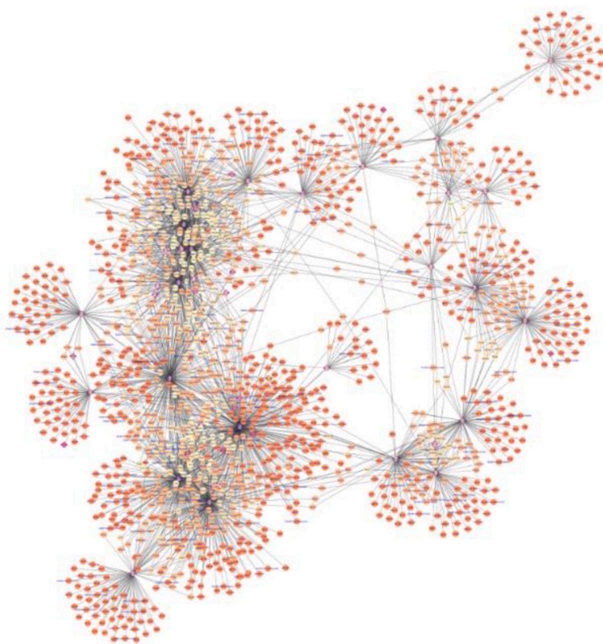

Fig. 4. Cotyledon regulatory network of differentially expressed genes and key transcriptions factors of beef heifers at day 83 of gestation. Nodes are differentially expressed genes or key transcription factors with a $|r| \geq 0.8$. Nodes with a magenta border were differentially connected between cotyledon and caruncle. The node size and color (from light to dark) are proportional to the number of connections for each gene. Nodes with few connections not linked to the main network are not showed. Transcription factors are represented by a diamond shape. High-resolution image is provided in supplementary material. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

interactions. Furthermore, complement and coagulation cascades, antigen processing and presentation, and phagosomes were over-represented in cluster D. The underlying genes of over-represented pathways (FDR < 0.05) for all clusters are reported in Table S2 – Table 5.

3. Discussion

The onset of placental attachment to the uterus is coordinated by a complex cross-talk of cellular and molecular processes. Likewise, orchestrated mechanisms regulate vasculogenesis, fetal growth and development, and maintenance of a successful pregnancy [13,24]. However, modeling fetal-maternal communication and the interplay of molecular mechanisms during early embryonic development and placentation is still largely unknown. Here, we report a comprehensive and systematic analysis of CAR and COT transcriptomic profiles from beef heifers at day 83 of pregnancy. We first identified up and down-regulated genes by comparing the expression profiles of the fetal (COT) vs. the maternal (CAR) placenta. Then, mining the data, we reported differentially expressed nutrient transporters and imprinted coding genes and putative TFs modulating gene expression differences between the tissues. Further, through co-expression and clustering analysis, we created regulatory networks to model the gene-gene relationship and tissue-specific gene rewiring. Finally, we pointed out over-represented BP and KEGG pathways that underlie tissue-specificities.

Our differential expression analysis showed that most of the DEGs were downregulated in COT, suggesting a pivotal role of the maternal portion of the placenta to establish and actively modulate this cross-talk. Involved in essential functions for the feto-maternal interaction, the DEGs underlined immune tolerance, trophoblast invasion, and tissue remodeling processes [25]. The functional analysis highlighted the different functions over-represented by the up and downregulated genes. Likewise, potentially activated and repressed pathways reinforced the specificities of tissue function. Moreover, transcriptional regulation through TFs and epigenetic mechanisms play a key role not only in fetal development but also in tissue communication. Our findings shed light on the differences in gene expression profiles between fetal and maternal tissues at the end of the first trimester of pregnancy. While we focused on female fetuses only, a growing number of studies have shown the interplay between the maternal condition and fetal sex on placental function and gene expression [26,27]. Therefore, further investigation is still required to understand the sex-depended placental differences in bovine.

3.1. Imprinted genes and non-coding RNAs play a role in placental function

By overlapping the DEGs with the list of imprinted genes from cattle, we identified 10 genes out of 14 reported as paternally imprinted in cattle. Imprinted genes have major effects on development and placental biology [10]. Here, *DIO3, DLK1, GNAS, IGF2, MEST, PEG10, PEG3,* and *RTL1* were among the upregulated genes in COT. Increased expression of paternally imprinted genes maximizes fetal resource extraction from the mother and improves offspring fitness [28,29]. These genes have been reported as regulating placental function and thus the supply of nutrients to the fetus [10,29]. For example, *PAG10* silencing [30] and *GNAs* mutation [31] were associated with reduced trophoblast differentiation and intrauterine growth retardation, respectively. Interestingly, miRNAs can be translated in clusters with imprinted genes. We found the *RTL1* gene upregulated in COT, and it is reported as paternally imprinted. *RTL1* is associated with a maternally-expressed antisense transcript (anti-RTL1), that serves as the primary transcript to generate miRNAs such as miR-431 and miR-433 in mice [32]. These miRNAs were found here as upregulated in COT. The paternal deletion Pol-like domain of *RTL1* led to late fetal and neonatal lethality and pre- and post-natal growth retardation of the mice embryo and placenta [33]. Our observations regarding the paternal contribution to the fetal portion of the placenta (COT) are supported by other authors [27,34] and leave open questions regarding the impacts of altered paternal conditions on placental development and function.

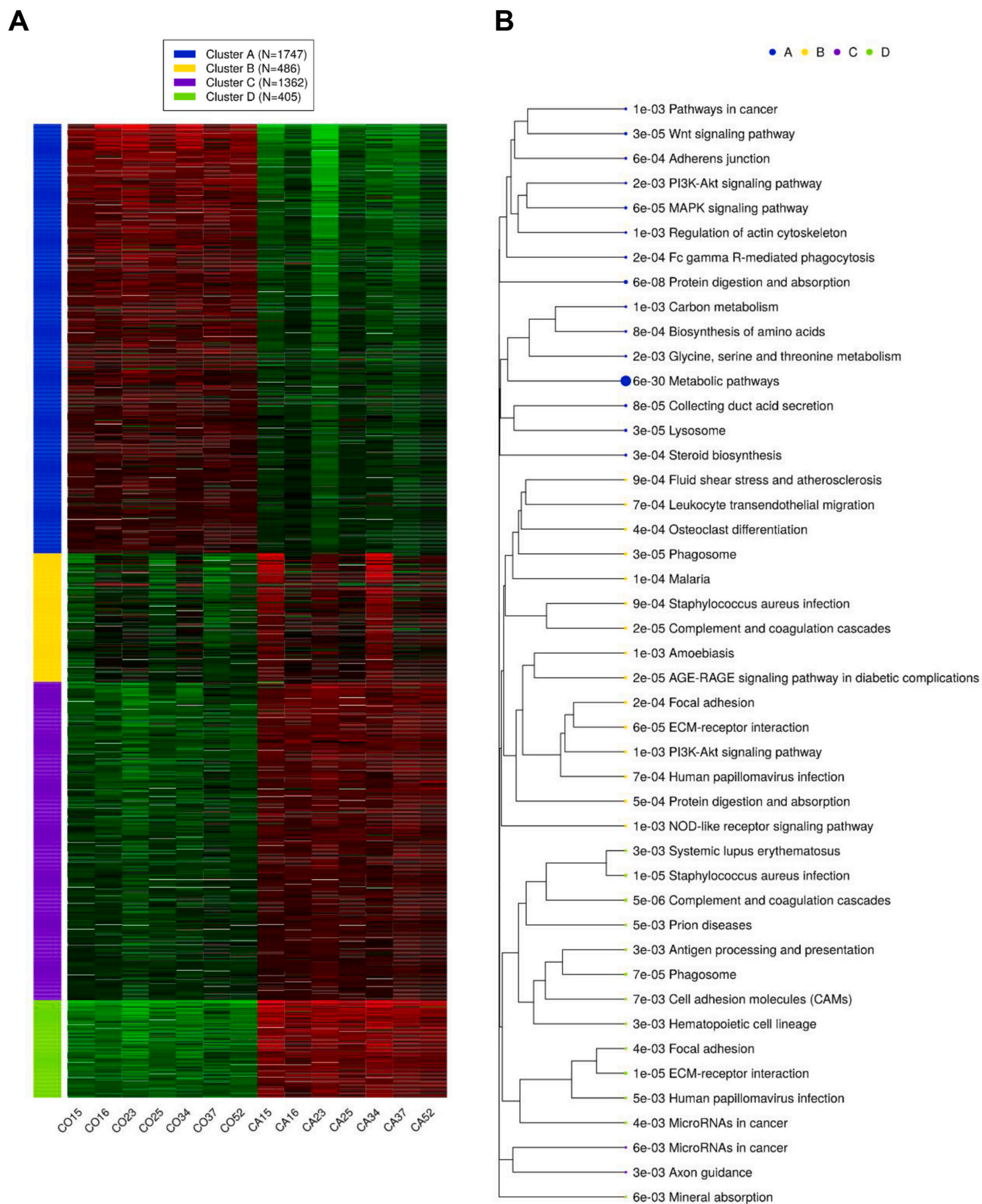

Fig. 5. Hierarchical clustering of gene expression (**A**) and over-represented KEGG pathways (**B**) of cotyledon (COT) and caruncle (CAR) genes from beef heifers at day 83 of pregnancy. Lower and greater expression levels than average for each row are color-coded as green or red, respectively. The *x-axis* shows COT (CO) and CAR (CA) sample ID. Clusters are color-coded according to the legend. The KEGG pathways are hierarchically arranged based on functional similarity. The bigger the dot, the more significant the term is (FDR $\leq$ 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Other potential epigenetic mechanisms modulating placental and fetal development found in our study were non-coding RNAs (ncRNAs). We identified 66 long and 22 small ncRNAs, including the upregulated bta-miR-433, miR-431, and miR-450b. Despite the gap in knowledge for the role of ncRNAs in placentation, they have been reported as pivotal for trophoblast cell functions and immune and inflammatory responses [6]. In mice, miR-450b was highly expressed and differentially regulated in trophoblast stem cells [35]. Although our approach was not designed to identify ncRNAs, these findings reinforce their roles in placental development and warrant further investigation.

3.2. Nutrient transporter-coding genes were differentially expressed

The interplay between the fetal needs and maternal nutrients available is tightly regulated [36,37]. Our approach identified several DEGs encoding nutrient transporters. Vitamin metabolic processes, biosynthesis of amino acids (AA), and steroid biosynthesis were over-represented by the upregulated genes in COT. Furthermore, nutrient-

sensing pathways, such as PI3K-AKT and MAPK signaling, were among the over-represented pathways from the highly expressed genes in COT. Among them, *RELN*, which is involved with glucose homeostasis, was found upregulated in COT.

Genes from the SLC family that mainly encode AA transporters were upregulated in COT, whereas some of the downregulated genes from the ABC and SLC families encoded lipid transporters. Nutrients are provided to the fetus initially as histiotrophic and then hemotrophic nutrition [38,39]. Menezes et al. [40] reported an increased concentration of neutral AA in the allantoic fluid at day 83 of pregnancy in response to maternal vitamin supplementation. During gestation, increased lipid transport is required to meet the fetal needs, for hormone synthesis, and as inflammatory mediators [41]. Considering the fetal and placental growth trajectories, an increased nutrient supply is expected to meet the demands of cellular turnover, differentiation, and placental development [39,40,42].

3.3. Transcription factors lead to tissue-specific gene regulation

Based on the RIF measure, we retrieved 84 TFs acting as potential modulators of differential expression. These TFs were then identified as hubs within the tissue network and differentially connected between tissues. Furthermore, although most of these TFs were not differentially expressed, a clear expression profile difference observed between tissues is likely driving the tissue specificities. Several TFs have been reported as required for placental development [3]. We identified nine genes from the FOX family as DEGs, most of them downregulated in COT. Furthermore, *FOXO4* was significantly more connected in CAR and pointed out as a key regulator of differential expression. The FOX family has been suggested as a central regulator of cell fate due to its role in modulating cell cycle and differentiation [43]. In addition to being highly expressed in the human placenta [4], *FOXO4* is essential for the neural differentiation of human embryonic stem cells [44].

We identified five members of the GATA family upregulated in COT. These genes are involved with gene expression regulation, trophoblast lineage differentiation, and trophectoderm maintenance [40,41]. Among them, *GATA2* and *GATA3* were both putative key regulators and differentially connected. Similar findings were found for *MSX1* and *HAND1* TFs. Ma et al. [45] reported that *GATA2* and *GATA3* were regulators of trophoblast-specific gene expression through the differential synthesis of placental hormones. From the MSX family of homeobox protein, we found *MSX2* upregulated in COT. Together, *MSX1* and *MSX2* are central regulators of tissue morphogenesis [46]. *MSX1* and *2* were involved with the embryonic development of limbs, craniofacial tissues [47], cardiovascular system, and fetal growth [48]. The *HAND1* gene is involved with placentation and cardiac morphogenesis [49]. Courtney et al. [50] reported that *HAND1* expression in placental chorion and trophoblast is necessary for labyrinth formation and vascularization. Furthermore, these authors found that *HAND1* knockout leads to early lethality in mice. Taken together, these findings shed light on the regulatory role of TFs in placentation, fetal-maternal cross-talk, and early embryonic development. Therefore, the rewiring of major regulators unveiled here suggests a differential modulation of gene expression, leading to tissue-specific functions essential to placenta development.

3.4. The transcriptomic profile of CAR and COT highlights tissue-specific functions

To shed light on the biological processes and KEGG pathways underlying the tissue-specific functions, we carried out not only the functional analysis of DEGs and TFs but also the clustered genes. Supporting the differential expression analysis findings, the *k-means* approach retrieved four clusters that highlighted the high and low gene expression between the tissues. Based on our findings, it seems that the Wnt pathway is repressed in COT at day 83 of pregnancy. Similar results were reported in buffaloes [13] and cattle [24]. The *DKK4* and *CTNNBIP1* genes that are negative regulators of Wnt [13,51] were upregulated in COT. Conversely, the positive regulator *WNT10A* [52] was downregulated. Other Wnt members, such as *WNT4* and *WNT7B* that were reported as important for the implantation process [53], were found upregulated in COT. Additionally, *WNT7A* was upregulated in COT while its targets were downregulated. The *WNT7A* gene is involved with the female Müllerian duct development and is a regulator of the *HOXA10* and *HOXA11* TFs, which modulate receptivity and implantation [54,55]. Altogether, the expression of canonical and non-canonical Wnt genes reinforces the role of this pathway in placental development during early gestation and suggests different regulatory mechanisms.

Another interesting observation was the changes in gene expression associated with tissue remodeling and structure. Tissue-related pathways over-represented in our study included ECM receptor interaction, focal adhesion, regulation of actin cytoskeleton, and adherens junction. The placentomal growth requires extensive tissue remodeling from early pregnancy to postpartum [2]. These changes seem to be more dramatic in CAR, as most of the genes underlying the aforementioned pathways were downregulated or less expressed in COT. Marked contrasting changes in gene expression patterns between COT and CAR included MMP and PAG families, which were down and upregulated in COT, respectively. PAGs are aspartic endopeptidases exclusively synthesized by the trophoblastic giant cells and are important mediators of processes related to maternal recognition of pregnancy [56]. Immunomodulatory actions and proteolytic activity have been suggested as potential roles of PAGs [57]. Lotfan et al. [13] reported increased PAG expression in the cotyledon of pregnant buffalos. Other gene families downregulated in COT included collagen, cadherins, integrins, and cathepsins. Together, these results pinpoint the key role of genes in remodeling the feto-maternal placental units.

In addition to the structural changes during placental development, maternal immune tolerance must be established for a successful pregnancy [58]. Among our findings, the *CD74* and *CFI* genes were downregulated in COT. These genes were suggested as responsible for maintaining an immunosuppressive environment to avoid the rejection of the fetus [13]. Conversely, cytokines with pleiotropic effects, such as IL-6 and IL-27, were upregulated in COT. Although IL-6 has been associated with tissue remodeling, hematopoiesis, and inflammation response [59], high levels of maternal IL-6 lead to maternal immune activation, which negatively affects fetal brain development [60]. On the other hand, IL-27 has been suggested as a regulator of local immune response and angiogenesis during pregnancy [58]. Further, supporting an immunosuppressive environment, we found genes coding the bovine leukocyte antigen (BOLA, BOLA-DRA, BOLA-DMA, and BOLA-DMB) and complement systems downregulated in COT. According to Dabrowska et al. [61], the BOLA system mediates immune tolerance during pregnancy. Likewise, the complement cascade is pivotal for a normal pregnancy, as its dysregulation has adverse effects for both the dam and the offspring [62]. Supporting the role of the DEGs in modulating the differences of immune function between CAR and COT, our approach retrieved several over-represented pathways, including complement and coagulation cascades, leukocyte transendothelial migration, phagosome, and antigen processing and presentation.

4. Conclusion

Based on a multi-tiered approach, our study revealed a complex and intricate gene network underlying the differences between fetal and maternal tissues. Furthermore, transcription factors play a key role in modulating the gene expression between tissues. Likewise, pathways related to nutrient transport, tissue differentiation and remodeling, and immune tolerance are pivotal for fetal-maternal cross-talk, successful implantation, and fetal development.

5. Material and methods

All experiments and methods complied with the ARRIVE guidelines and were approved by the North Dakota State University Institutional Animal Care and Use Committee (IACUC A19012).

5.1. Animals, tissue collection, and RNA isolation

The *gravid uteri* of seven pregnant Angus-cross heifers carrying female fetuses were collected through ovariohysterectomy at day 83 ± 2 of gestation. This time point is the end of the first trimester, and the placenta is still growing exponentially [63]. Likewise, there is an increased gene expression related to the still increasing fetal nutrient demand and the still developing placental structure [64]. Additionally, fetal organogenesis has already finished and the secondary myogenesis is beginning [65]. Thus, the first trimester is critical not only for placental development and later "functional capacity" of the placenta but also for fetal development.

The cotyledon (COT) and caruncle (CAR) from a large placentome close to the fetus were manually dissected [42], snap-frozen, and stored at −80 °C. The heifers used in this study were part of the control group (NoVTM_LG) of a much larger experiment described elsewhere [40,64].

Total RNA was isolated from COT and CAR tissues using the RNeasy® kit (Qiagen®, Germantown, MA, USA) followed by on-column DNase treatment, according to the manufacturer's protocol. The Agilent 2100 Bioanalyzer and agarose gel electrophoresis were used for mRNA quality control. Only samples with RIN values greater than 6 were sequenced (RIN average = 7.8).

5.2. Sequencing and data analysis

Sequencing and data quality control were performed as previously described by Diniz et al. [64]. Briefly, strand-specific RNA libraries (n = 14, 7 per tissue) were prepared using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (New England BioLabs®, Ipswich, MA, USA). Paired-end 150-bp reads at a depth of 20 M reads/sample were sequenced on the Illumina® NovaSeq 600 platform. Library preparation and sequencing were performed at Novogene Co. (Nanjing, China).

Prior to statistical analysis, raw reads were assessed for quality control using FastQC v0.11.8 [66] and MultiQC v1.9 [67]. Reads with a PhredScore lower than 30, sequencing adaptors, low-complexity reads, and reads containing low-quality bases were filtered out. Read mapping was based on the ARS-UCD 1.2 *Bos taurus* reference genome and the gene annotation file (release 100) retrieved from the *Ensembl* database. The raw counts per gene were retrieved with the *–quantMode* GeneCounts option from the STAR aligner v. 2.7.3a [68] while mapping. Post-mapping quality control was performed using MultiQC, NOISeq v.2.26.0 [69], and edgeR v.3.24.0 [70] software.

5.3. Differential expression analysis and identification of key transcription factors

Read counts from STAR were used as input for data quality control and differential expression analysis performed on the RStudio v.1.1.442 environment for R v.3.5.1. [71,72]. The *filterByExpr* function from the R-package edgeR was used to filter out lowly or not expressed genes. Genes with expression values lower than 1 count per million in 50% of the samples were filtered out.

Further, differential gene expression analysis was performed using DESeq2 v.1.22.1 [73] by contrasting COT vs. CAR. As the heifers used in this trial were born in two different farms, we used a multi-factor design (~ farm + tissue) when performing DESeq2 analysis. *P*-values were adjusted (*padj*) based on the Benjamini–Hochberg procedure for false discovery rate (FDR) implemented in the DESeq2 package. Genes were considered differentially expressed when the FDR ≤ 0.05 and abs (log2FC) ≥ 1.

The Regulatory Impact Factor (RIF) algorithm RIF1 and RIF2 [17] were used to identify TFs potentially regulating gene expression between CAR and COT tissues. The RIF algorithm was implemented based on the source code available from Reverter et al. [17] and as described by Diniz et al. [74]. To identify putative regulators, 1396 bovine TFs were downloaded from the Animal Transcription Factor Database (Animal TFDB v3.0) [18]. The list of TFs was overlapped with the genes from the CAR and COT datasets and those not expressed were filtered out. After filtering, 863 TFs were contrasted to the list of DEGs, comparing COT vs. CAR tissues. TFs with RIF1 or RIF2 z-scores |1.96| were considered significant [17].

5.4. k-means clustering and co-expression network analyses

An unsupervised clustering approach was adopted to identify expression patterns in the data and group genes with similar behavior between tissues. To this end, the gene count data was normalized through the *VST* function from DESeq2 and used as input for the iDEP v.9.2 [75]. In the clustering analysis, 14,312 genes were sorted by standard deviation (SD), and the top 4000 were selected (SD ≥ 0.4). Genes were mean-centered and then hierarchically clustered using Pearson's correlation as distance metrics. Data exploratory analysis and visualization were performed on iDEP as well.

The co-expression profile of gene pairs for CAR and COT tissues was created based on the partial correlation and information theory (PCIT) algorithm [22]. For gene network inference, the DEGs and the significant key TFs were used to create CAR and COT networks separately. Significantly co-expressed pairs were selected when a TF was present and showed a partial correlation greater than |0.8| ($p < 0.05$). Hub genes were selected based on the connectivity (K) (2 SD from the mean, $p \leq 0.05$) retrieved from the Cytoscape Network Analyzer tool v.2.79 [76], as reported elsewhere [74]. Cytoscape v.3.8.2 [77] was used for network visualization.

To explore the differences in gene connectivity between tissues, the DK for each gene in the networks was measured [74]. The K for each gene was standardized by dividing the gene connectivity by the maximum connectivity in the network [23]. Then, the DK index was defined as $DK_i = KCAR_{(i)} - KCOT_{(i)}$. The DK index was transformed into a z-score, and values located ±1.96 SD from the mean were considered significant ($p \leq 0.05$).

5.5. Functional over-representation analysis

To gain biological insights on the roles of the DEGs, TFs, and co-expressed genes, a functional over-representation analysis was performed. A three-tiered approach was taken to detect differences in BP and KEGG pathways involved in the CAR and COT function. First, the WebGestalt web tool [78] was used for BP functional analysis of up and downregulated genes separately. Second, a cluster analysis framework based on the ClueGo v.2.5. 7 [19] plug-in for Cytoscape retrieved the KEGG pathways underlying DEGs and TFs. Finally, functional analysis of gene clusters identified through *k-means* was performed using iDEP. Significant over-represented BP and KEGG pathways were identified after *p*-value multiple testing adjustments (FDR ≤ 0.05). The *B. taurus* annotation was used as background for over-representation analysis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2022.110274.

Data availability

All relevant data are within the paper and its Supplementary Information files. All RNA-sequencing data is publicly available on NCBI's Gene Expression Omnibus through GEO Series accession number GSE165378 (Bioproject PRJNA694189). All additional datasets generated and analyzed during this study are available from the

corresponding author.

Author statements

Conceptualization, W.J.S.D., C.R.D., K.L.M., J.S.C., J.C.F. and R.R.S.; Animal management, K.L.M., C.J.K., F.B., J.D.K. and K.K.S.; sample collection; L.P.R., P.P.B., A.K.W., T.L.N. and S.T.D.; RNA isolation, S.T. D.; bioinformatic analysis, W.J.S.D., writing-original draft preparation, W.J.S.D.; writing-review and editing, all authors; supervision, A.K.W. and C.R.D.; funding acquisition, L.P.R., C.R.D., K.L.M. and J.S.C. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

Authors J. C. Forcherio and R. Scott are employees of Purina Animal Nutrition LLC (Land O'Lakes, Inc., Arden Hills, MN, USA), which sponsored the sample analysis for this experiment. Purina Animal Nutrition LLC manufactured the Purina® Wind & Rain® Storm® All-Season 7.5 Complete mineral, the VTM and NoVTM pellets, and the protein/energy supplement used in this study. The funders had no role in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results that were conducted entirely independently by North Dakota State University personnel. The first author led the writing of the paper with inputs from the co-authors, who declare no conflict of interest.

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