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Characterization of Choline Transporters in the Human Placenta over Gestation

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Abstract

INTRODUCTION—The developing fetus relies on the maternal blood supply to provide the choline it requires for making membrane lipids, synthesizing acetylcholine, and performing important methylation reactions. It is vital, therefore, that the placenta is efficient at transporting choline from maternal to fetal circulation. Although choline transporters have been found in term placenta samples, little is known about what cell types express specific choline transporters and how expression of the transporters may change over gestation. The objective of this study was to characterize choline transporter expression levels and localization in the human placenta throughout placental development.

METHODS—We analyzed CTL1 and −2 expression over gestation in human placental biopsies from 6 to 40 weeks gestation (n=6–10 per gestational window) by immunoblot analysis. To determine the cellular expression pattern of the choline transporters throughout gestation, immunofluorescence analysis was then performed.

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AUTHOR CONTRIBUTIONS

HKB and VDW designed and directed experiments, with significant intellectual input from RGR. HKB and KMT performed Western blot, immunofluorescence, and image analysis. CEG, and AP performed immunofluorescence. TP performed the microarray database analysis. VDW provided tissue samples and expertise in the interpretation of the data. HKB wrote the manuscript, with critical review and edits by VDW and RGR. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

RESULTS—Both CTL1 and CTL2 were expressed in the chorionic villi from 6 weeks gestation to term. Labor did not alter expression levels of either transporter. CTL1 localized to the syncytial trophoblasts and the endothelium of the fetal vasculature within the chorionic villous structure. CTL2 localized mainly to the stroma early in gestation and by the second trimester co-localized with CTL1 at the fetal vasculature.

DISCUSSION—The differential expression pattern of CTL1 and CTL2 suggests that CTL1 is the key transporter involved in choline transport from maternal circulation and both transporters are likely involved in stromal and endothelial cell choline transport.

Keywords

Choline transport; CTL1; CTL2; chorionic villi; trophoblasts; fetal endothelium

INTRODUCTION

Choline is an essential nutrient that must be obtained through the diet for maintenance of proper health. The synthesis of phosphatidylcholine and sphingomyelin, major constituents of cell membranes that comprise the majority of total membrane lipid content, require choline [1]. Choline is also a critical precursor of the synthesis of the neurotransmitter acetylcholine [2]. Additionally, choline provides a source of methyl groups for the synthesis of S-adenosylmethionine (SAM), the primary methyl donor for cellular methylation reactions [3]. With choline playing roles in such critical pathways in cellular biology, it is not surprising that a choline deficient diet would have adverse effects on health. In fact, several investigators have shown that lack of adequate choline levels can lead to development of fatty liver [4, 5], liver damage [6], muscle damage [7], and increased sensitivity to carcinogens [8] in adult humans. Additionally, Niculescu [9] and Mehedint [10] have used rodent models to demonstrate a loss in fetal hippocampal progenitor cell proliferation and angiogenesis, respectively, resulting from a maternal diet deficient in choline during pregnancy. These results demonstrate that choline not only plays an important role in adult health, but also plays an essential role in fetal brain development. In 1998, the National Academy of Sciences recognized choline as an essential nutrient and recommended daily intake levels (550 mg/day for men, 425 mg/day for non-pregnant and 450 mg/day for pregnant women).

Enriching adult diets with choline has been shown to improve cognition [11–13]. Importantly, enriching the diet of pregnant mothers with choline has been shown to have beneficial consequences for offspring that can last well into adulthood. Rodent models have demonstrated that supplementing the diets of dams with choline can improve spatial and temporal memory in offspring [14] and can improve cognitive function for years after birth [15]. Recently, this phenomenon has been demonstrated in humans. Infants from mothers supplemented with phosphatidylcholine, as a choline source, during pregnancy showed improved cerebral inhibition as measured by electroencephalographic P50 ratio [16]. These observations have important implications in understanding the origins of psychotic disorders. Abnormal development of GABA-related inhibitory neurocircuitry has been implicated in a variety of neuropsychiatric illnesses including externalizing disorders (ADHD and conduct disorder), anxiety disorders (including PTSD), psychotic illnesses

(including schizophrenia and bipolar), depressive disorders, and substance use disorders [17–22]. Toward the end of gestation, stimulation of the α7 nicotinic acetylcholingergic receptor facilitates the transition of the neurotransmitter GABA from excitatory to inhibitory. During fetal development, acetylcholinergic innervation of most brain regions has not yet fully developed; choline itself is the primary prenatal agonist at the alpha 7-nicotinic acetylcholine receptor and thus sufficient choline is critical for proper development of cerebral inhibitory neurons. Choline availability during fetal development may have a crucial role in brain development and deficiencies in choline may lead to lasting effects in the offspring.

During development, the fetus depends on maternal supply of choline for its metabolic needs [23]. The placenta plays a vital role in the active transfer of choline from the maternal blood supply to the fetus. It was demonstrated in 1985 [24], through perfusion studies in the guinea pig, that choline transport in the placenta is rapid, unidirectional against a concentration gradient, occurs at both the maternal and fetal interface of the placenta, and is sodium-independent. In 1994 [25], a similar choline transport mechanism was demonstrated in brush border vesicles isolated from human term placenta and in 2005 [26], a similar mechanism was demonstrated in human BeWo and JEG-3 trophoblast cell lines derived from choriocarcinomas. Further characterization of choline transport in the placenta has been limited.

Choline transport is known to occur through three different protein-mediated transport systems: (1) the high affinity choline-specific CHT1 transporter (SLC5A7) that is expressed in specific regions of the brain rich in cholinergic neurons, (2) the intermediate affinity choline transporter-like CTL proteins (SLC44A1–5) found in multiple tissues, and (3) the low affinity organic cation OCT transporters found in various tissues [27]. The objective of this study is to characterize choline transporter expression levels and localization in the placenta over gestation. The chorionic villi are the primary sites of placental nutrient transport and were, therefore, the focus of our studies. Choline transporter expression levels were examined from chorionic villi lysates at various time points throughout gestation. Corresponding tissue sections were examined by immunofluorescence to determine specific cell types expressing the transporters.

METHODS

Placenta Samples

Chorionic villi were collected from human placenta at various gestational time points and grouped into 6–8 weeks (n=6), 10–12 weeks (n=6), 18–24 weeks (n=6), 28–32 weeks (n=6) and, 37–40 weeks both with (n=10) and without (n=10) labor. Collection of placenta was approved by the Institutional Review Board of the University of Colorado and informed consent was obtained. Subjects were excluded if mother had chronic disease, was a smoker, or abused alcohol or drugs. First and second trimester placenta were collected from uncomplicated elective terminations. Third trimester preterm placentas were collected from subjects with preterm labor that were normotensive and did not have preterm premature rupture of membranes (PPROM) or chorioamnionitis. Term placentas were collected from uncomplicated vaginal delivery and unlabored C-section. Tissue was immediately snap

frozen in liquid nitrogen or placed in formalin (described in more detail below) for analysis of protein expression and localization.

Western Blot Analysis

Tissue was briefly washed in cold phosphate buffered saline (PBS) before being snap frozen in liquid nitrogen and stored in −80°C. Frozen tissue was placed in ice-cold lysis buffer (Tris-HCl, pH 7.4 50 mM, NaCl 150 mM, NP-40 1%, Sodium deoxycholate 0.5%, SDS 0.1%, EDTA 5 mM; RIP A Buffer with EDTA, Boston Bioproducts, Ashland, MA) with protease inhibitors (Complete EDTA-free Protease Inhibitor Cocktail, Roche, Mannheim, Germany) and homogenized with a Brinkman Instruments Polytron, centrifuged at 14,000 rpm for 10 minutes at 4°C, and supernatant collected. Tissue lysates (15 μg total protein) were denatured, resolved on 10% SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio Rad, Hercules, CA, USA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for one hour at room temperature (RT) before treatment with either rabbit anti-human SLC44A1/CTL1 (1:1000, Bioss Inc., Woburn, MA, USA), mouse anti-human SLC44A2/CTL2 (1:500; Sigma, St. Louis, MO, USA), or rabbit anti-human β-actin (1:10,000; Abcam, Cambridge, MA, USA) overnight at 4°C. Membr anes were then washed 4 times with TBST for 15 minutes before treatment with horseradish peroxidase-conjugated goat anti-rabbit (1:10,000; GE Healthcare, Buckinghamshire, UK) or goat anti-mouse (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) antibodies for 1 hour at room temperature. Membranes were washed with TBST as described above and then visualized using an ECL Prime Western Blotting Detection Reagent (GE Healthcare) and X-ray film (CL-XPosure Film, Thermo Scientific, Rockford, IL, USA). Densitometry was performed using ImageJ software (Wayne Rasband, National Institutes of Health), normalizing relative band density to the loading control.

Immunofluorescence

Chorionic villi were collected from human placenta at the gestational time points described above. Tissue was fixed in 10% buffered formalin at RT for 24–48 hours before being embedded in paraffin and sectioned. Tissue sections were hydrated with washes in xylene (2 twenty minute washes) followed by decreasing amounts of ethanol (2 washes in 100% ethanol, one wash in 70% ethanol, and one wash in 30% ethanol) for three minutes each. Sections were then washed in PBS two times for 5 minutes each before antigen retrieval, by boiling sections in citrate-based Antigen Unmasking Solution (Vector Laboratories, Inc., Burlingame, CA, USA) for 10 seconds followed by a 45 second rest and repeated 10 times. Sections were cooled for 10 minutes before washing with PBS and permeabilizing with 1% Triton X-100 for 15 minutes. After two washes with PBS, sections were blocked for one hour with 10% normal donkey serum plus 10 μM/ml saponin. Sections were then treated with rabbit anti-human SLC44A1/CTL1 (1:200, Bioss Inc., Woburn, MA, USA), mouse anti-human SLC44A2/CTL2 (1:100; Sigma, St. Louis, MO, USA), mouse anti-human CK7 (1:50, Dako, Carpinteria, CA, USA), rat anti-human CK7 (1:50, kind gift from M. McMaster, University of California, San Francisco, USA), mouse anti-human CD31 (1:100, Abcam, Cambridge, MA, USA), and/or rabbit anti-human CD31 (1:200, Abcam) overnight at 4°C. After being washe d five times with PBS, five minutes each, sections were treated

with donkey anti-rabbit-CY3 (1:100, Jackson ImmunoResearch), donkey anti-mouse-488 (1:100, Jackson ImmunoResearch), and/or donkey anti-rat-FITC (Jackson ImmunoResearch) for 45 minutes in the presence of DAPI (DAPI (5 μg/ml, MP Biochemicals, Solon, OH, USA). Sections were then washed with PBS (5 times, 5 min each) before application of OPDA (20 mg/ml, o-phenylenediamine dihydrochloride in 1M Tris, pH 8.5) to preserve fluorescence and coverslip applied. Specificity of antibodies was tested as described above and results are shown in Supplementary Figures 2 & 3. Fluorescence was imaged using an Olympus Spinning Disk confocal microscope located in the University of Colorado Advanced Light Microscopy Core Facility. Images were analyzed using Slidebook Software (Intelligent Imaging Innovations, Inc., Denver, CO, USA).

STATISTICS

Data are presented as Mean ± Standard Error of the Mean (s.e.m.). A one-way ANOVA was used to determine variance among multiple gestational groups, with a Tukey's Multiple Comparison post-test to determine significance between individual groups. A p value of < 0.05 was considered significant.

RESULTS

Choline transporter gene expression in human term placenta

To determine the major choline transporters expressed in the human placenta, we first analyzed the gene expression profiles of the known choline transporters in an established microarray database [28]. This analysis revealed that CTL1, CTL2, and OCT3 were the only transporters that showed Affymetrix intensity values above background. CTL1 and CTL2 showed the highest intensity values, suggesting these are the most highly expressed choline transporters (Supplementary Figure 1). Although OCT3 is expressed in the placenta, Kekuda and colleagues [29] have shown that this organic cation transporter does not transport choline to any significant level, and likely plays a role in the placenta independent of choline uptake. Therefore, we focused on characterizing CTL1 and CTL2 expression in the human placenta.

CTL1 and CTL2 protein expression levels over gestation

To determine whether CTL1 and CTL2 protein expression changes over gestation, we performed Western blot analysis on tissue lysates from chorionic villi isolated from placenta collected at specific gestational windows (6–8 weeks, 10–12 weeks, 18–24 weeks, 28–32 weeks, term with labor, term without labor). CTL1 protein expression appears to be consistently expressed throughout gestation (Figure 1), with no drastic changes in expression levels. Importantly, it appears CTL1 expression levels maintain consistency even when the placenta is exposed to the dynamic environment of labor. In the term placenta, the presence of a lower molecular weight band (~38 kDa) appears in a few samples regardless of labor status. Although this lower molecular weight band has been observed by other investigators [30, 31] and did not appear in our IgG and antigen peptide controls (see Supplementary Figure 2), the significance of this band is unkonwn. Therefore, we did not include this band in our densitometry analysis. CTL2 protein expression is not as consistent

as CTL1. Early in gestation, CTL2 expression level is low and is higher after 10 weeks gestation, although the increase in protein expression is not significant with the current sample size. At term, CTL2 protein expression does not seem to alter significantly with the presence or absence of labor.

Localization of CTL1 and CTL2

To determine where CTL1 and CTL2 are localized in the placenta and whether localization changes during development, we performed immunofluorescence on placenta tissue sections. The uptake of choline by the placenta from the maternal blood supply is at the chorionic villi. Therefore, the localization of CTL1 and CTL2 within the chorionic villous structure was examined by immunofluorescence on placenta tissue collected at specific time points throughout gestation. Figure 2A shows the general localization of CTL1 and CTL2 in representative samples from early (6–8 weeks), mid (18–24 weeks), and late (39–40 weeks) gestation. CTL1 appears to localize most strongly to the syncytial trophoblasts that form the outer layer (adjacent to the maternal blood) of the chorionic villi. There is also a consistent presence of CTL1 in the stroma. As pregnancy progresses, CTL1 expression is noted in ring-like structures which appear to correspond with the fetal vasculature. CTL2, on the other hand, does not appear in the trophoblast layer(s). Instead, CTL2 appears to localize most strongly within the stroma and as pregnancy progresses appears to localize within the vascular-like structures within the villi. The pattern of localization for both CTL1 and CTL2 is consistent during all gestational windows examined (6–8 weeks, 10–12 weeks, 18–24 week, 28–32 weeks, 39–40 weeks, data not shown for 10–12, 28–32 week time points).

To determine how CTL1 and CTL2 were distributed within the distinct villous structures indicated above, we examined the chorionic villi at higher magnification (Figure 2B & C). At the early gestational time points, CTL1 is diffusely distributed throughout the trophoblast cells that make up both the cytotrophoblast and snycytiotrophoblast layers of the villi. By 18–24 weeks, CTL1 expression remains high in the trophoblasts and is now also clearly localized to vascular-like structures within the villi. At 28–32 weeks, CTL1 shows a strong localization within the vascular-like structures. Interestingly, CTL1 expression in the syncytial trophoblasts becomes strongly associated with the apical region/membrane at this time point. Throughout gestation, CTL1 can also be found in stroma of the chorionic villi. In contrast to CTL1, CTL2 does not localize to trophoblasts at any time during gestation. Early in gestation, CTL2 is found mainly in the stroma. At 18–24 weeks, CTL2 starts to appear in the vascular-like structures, where co-localization with CTL1 can begin to be visualized. By 28–32 weeks, CTL2 shows a strong localization in the vasculature. At term, CTL1 and CTL2 localization remains as seen at 28–32 weeks, with CTL1 mainly in the trophoblasts and vascular-like structures and CTL2 mainly in the vascular-like structures and stroma. When localization was compared between term chorionic villi of placentas from labored versus non-labored subjects, neither transporter changes localization with the physiologic process of labor (Figure 2C).

Co-localization with trophoblasts and endothelial markers

To confirm that CTL1 is localizing with trophoblasts, sections of chorionic villi, at all gestational time points were treated simultaneously with antibody directed to CTL1 and

antibody directed to the trophoblast marker CK7. Fluorescence from the secondary antibodies revealed that, indeed, CTL1 co-localized with CK7 (Figure 3). CTL2, however, did not co-localize with CK7 at any gestational time points.

To confirm that CTL1 and CTL2 localize to the fetal vasculature, sections of chorionic villi, at all gestational time points, were treated simultaneously with antibody directed to either CTL1 or CTL2 and antibody directed to the endothelial cell marker CD31. Figure 4 shows that both transporters co-localize with CD31 in the chorionic villi at gestational time points where fetal vasculature is present.

DISCUSSION

This is the first study that has examined human placental choline transporter, specifically CTL1 and CTL2, expression and localization over gestation. We observed constitutive expression of CTL1 throughout placental development. CTL1 was found in the cytotrophoblasts and syncytiotrophoblasts of the chorionic villi from early gestation (around 8 weeks) through term (around 40 weeks). Toward the end of the first trimester, CTL1 also showed a strong expression in the endothelium of the fetal vasculature. CTL2 showed a more variable expression pattern. Early in gestation, CTL2 was found mainly in the stroma of the chorionic villi. By 18 weeks, CTL2 could be found in the endothelium of the fetal vasculature of the chorionic villi and became strongly expressed in the endothelium by 28 weeks. These observations suggest that CTL1, being present in the syncytial trophoblasts, is likely the main choline transporter responsible for placental uptake of choline from the maternal blood supply. CTL2 expression levels correspond to the growing vasculature of the placenta and the strict localization of CTL2 to the endothelium suggests that CTL2 may play a specific role in endothelial choline transport.

CTL1 was localized to the apical region of the syncytial trophoblasts, as would be expected for choline uptake from the maternal blood. However, CTL1 was also found in the cytosol. This pattern of expression could represent cycling of the transporter between the plasma membrane and the cytosol for regulation of choline transport. It has been reported that movement of CTL1 out of the membrane reduces choline transport activity in macrophages [32]. Yuan and colleagues [30], when characterizing the promoter region of the CTL1 isoforms, also suggested that CTL1 may be found in the membranes surrounding intracellular organelles for the shuttling of choline from the plasma membrane to the mitochondria, for oxidation of betadine, and the nucleus, for utilization in the phosphatidylcholine cycle. Therefore, it is expected that intracellular expression of CTL1 could have a functional role in choline transport.

Constitutive expression of CTL1 in the syncytial trophoblasts of the chorionic villi is in agreement with work from Lee and colleagues [33] where they showed that CTL1 was the main choline transporter in the rat syncytial trophoblast cell line TR-TBT. They were able to demonstrate that the CTL1 transport activity in TR-TBT cells was similar to what had been previously described for human placenta, weakly sodium-dependent, saturable, and inhibited by hemicholinium-3 (HC-3). Of note, it has been demonstrated that CTL1 has 2 splice variants, CTL1a and CTL1b, which have been detected in human term placenta tissue

lysates. Our studies, however, are unable to determine if the ratio of splice variants change over gestation.

In contrast to our observations, Yara and colleagues [34] have recently shown expression of CTL2 in the trophoblastic cell line JEG-3 and suggest a potential role for CTL2 in the low-affinity choline transport found in these cells [26]. The discrepancy between results is likely due to the approach of analysis. We examined choline transport in native tissue from normal placentas and Yara investigated choline transporters in a choriocarcinoma cell line. Cancer cells are known to have aberrant choline transporter expression and activity [27] and, therefore, may explain differences in CTL2 expression seen in JEG-3 cells and our normal placenta tissue. Alternatively, it may be that expression of CTL2 in the trophoblasts of the tissue samples examined in this study may be too low to detect by our Western and IP techniques.

Although this is the first report of the CTL2 expression pattern in the placenta, others have shown that CTL2 has 2 isoforms driven off of different promoters. It is interesting to note that only the P2 isoform of CTL2 (CTL2-P2) has choline transport activity in other tissues [35]. Our studies were not able to distinguish the 2 isoforms, CTL2-P1 and CTL2-P2, so how much CTL2 contributes to choline transport in the placenta remains to be determined. Since the expression pattern of CTL2 does not follow the same pattern as CTL1, CTL2 may serve an independent role from CTL1 and is not just simply a redundant molecule.

To our knowledge this is the first report of CTL proteins localizing to the placental vasculature. CTL1 and CTL2 are both strongly expressed in endothelial cells, suggesting a significant need for choline in these cells. Whether this is unique to the fetal vasculature of the placenta is an interesting question. Kommareddi [35] has shown both membrane and cytosolic staining of CTL2 in other tissues (in rodent), including keratinocytes, nerve cells, kidney and spleen, but their analysis did not include the placenta.

Western blot analysis of CTL expression and immunofluorescence analysis of CTL localization at term showed that the process of labor does not significantly change CTL1 or CTL2 expression levels or localization. The placenta is exposed to oxidative and inflammatory stresses during labor [36] and microarray analysis of placenta from women who delivered vaginally (labored) shows over 300 genes that differed in expression pattern from placentas from women who underwent elective cesarean section (unlabored) [37]. Although labor produces significant changes in the placenta, our data suggests that CTL transporters are not regulated or altered by these changes. CTL transporters, however, have been shown to be increased by glucocorticoids. Nakamura et al. [38] demonstrated that treatment of human alveolar type II cells, A549, with dexamethasone can increase CTL1 and CTL2 expression and can enhance choline uptake in these cells. Our placenta samples that were exposed to the corticosteroid betamethasone (preterm samples) did not suggest a dramatic increase in choline transporter expression, but number of samples and variable time from betamethasone exposure are not adequate to fully address this relationship in the current study.

In conclusion, we have demonstrated that CTL1 and CTL2 are expressed in the chorionic villi of human placenta throughout gestation. CTL1 is expressed in the syncytial trophoblasts and cytotrophoblasts and the endothelium of the fetal vasculature. CTL2 is expressed in the stroma during early gestation and can be found strongly expressed by the fetal endothelium as placental development progresses. This change in expression may reflect, at least in part, the increase in fetal vascular development. It will be important for future studies to determine the contribution of each transporter, particularly each isoform of the transporters, to choline transport in both syncytial trophoblasts and endothelial cells. The CTL1 and CTL2 transporters have known coding region SNPs (rs3199966;S644A, rs2288904;Q154R, respectively), but the functional significance of these variants is unknown. Determining the impact of these SNPs on transport capacity will be needed to assess the predictive power genetic analysis would have related to fetal choline availability. Understanding how the placenta contributes to the uptake and fetal delivery of choline, an essential and critical nutrient for fetal growth, development and health outcome, during gestation holds promise in defining potential opportunities for interventions that could improve fetal outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- CTL1 and CTL2 show different expression patterns in the placenta over pregnancy.
- CTL1 localizes to syncytiotrophoblasts and endothelium of chorionic villi.
- CTL2 localizes to fetal endothelium of chorionic villi.
- Labor does not affect expression levels or localization of CTL1 or CTL2.

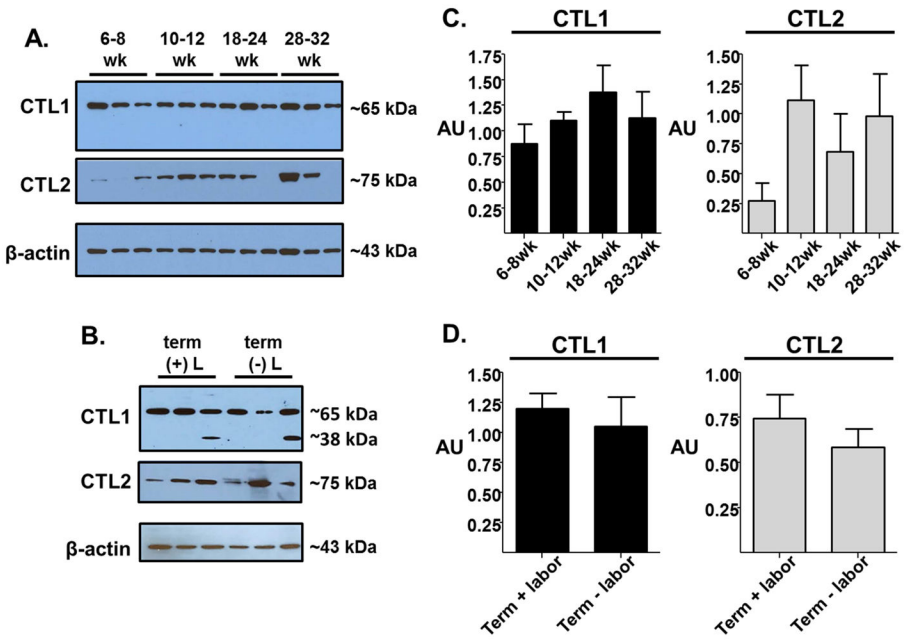
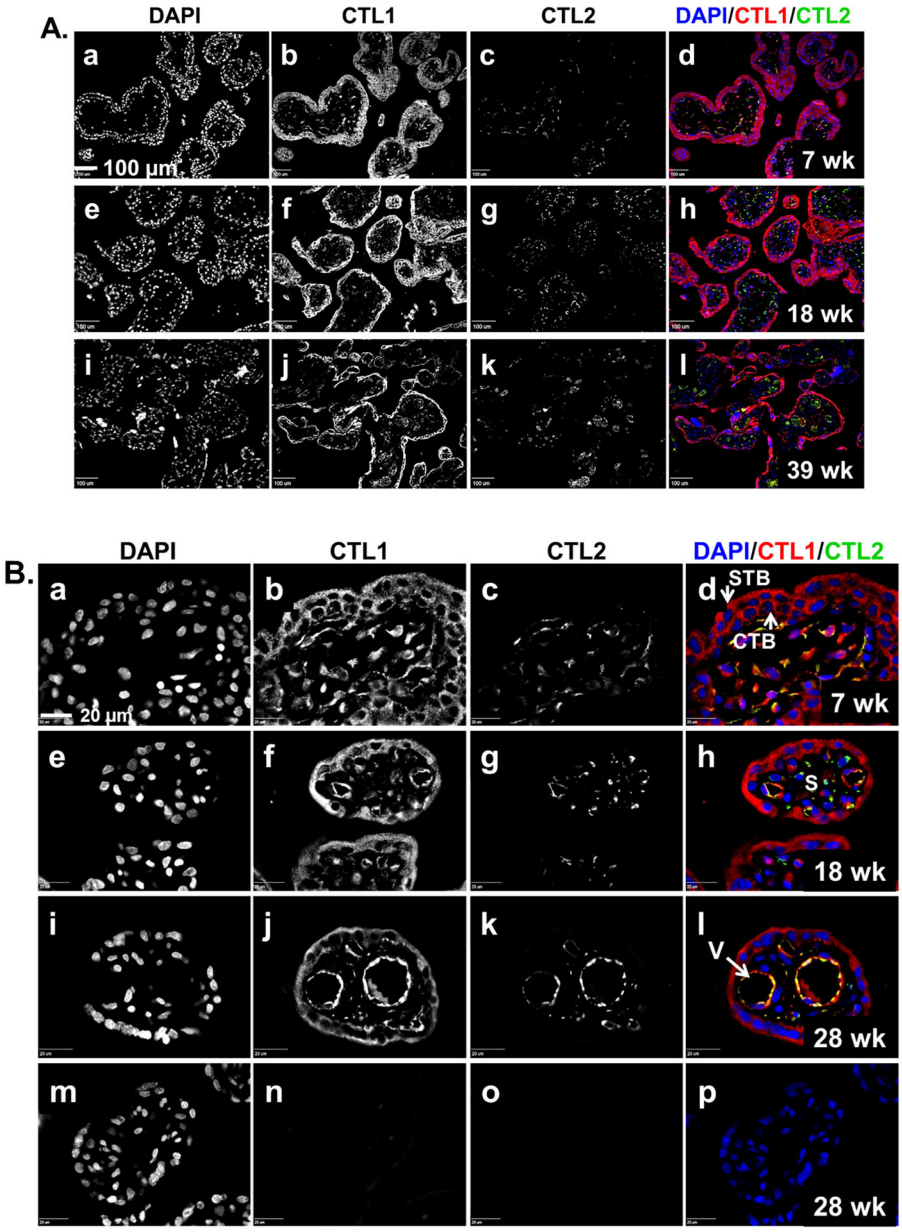


Figure 1. Placental CTL1 and CTL2 expression levels over gestation

Western blot analysis of CTL1 and CTL2 protein expression was performed using human placenta lysates of the chorionic villi region at 6–8, 10–12, 18–24, 28–32, and 37–40 (labored and unlabored) week gestation. (**A** & **B**) Representative blots for CTL1 and CTL2 protein expression levels for three samples at each gestational time point with β-actin serving as a loading control. (**C** & **D**) Western blot densitometry from multiple western blot analyses was performed using ImageJ. Normalization across blots was done on identical placental sample present on each blot. Mean ± s.e.m., n=5–13 per gestational group.



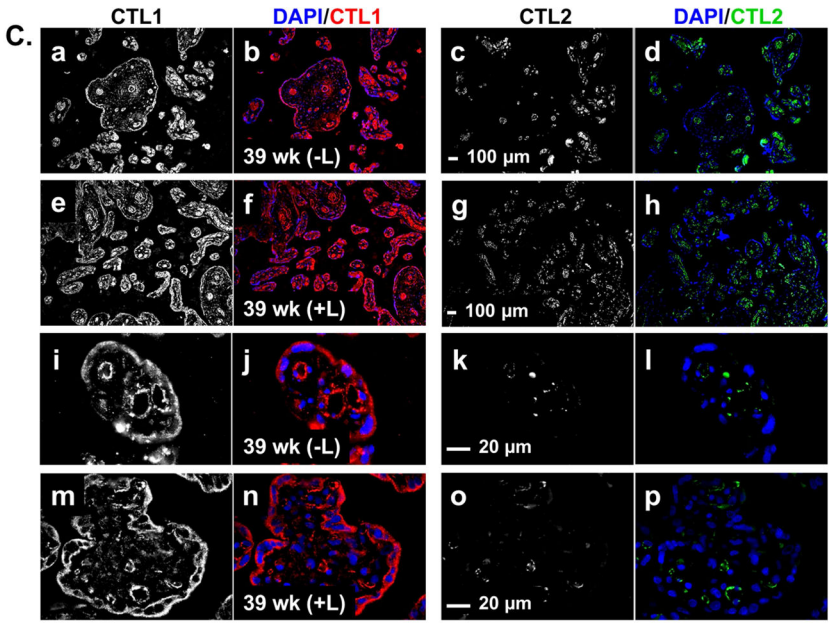


Figure 2. CTL1 and CTL2 localization in chorionic villi

Immunofluorescence was performed on paraffin embedded tissue sections from the chorionic villi region of human placenta collected at 6–8, 10–12, 18–24, 28–32, 37–40 (labored and unlabored) week gestational time points. Representative images for gestational windows are displayed. Fluorescence from antibodies directed to CTL1 (red) and CTL2 (green) was imaged with a confocal microscope. Nuclei are stained with DAPI (blue). (**A**) Lower magnification (scale bar: 100 µm) representative images showing localization of nuclei (a, e, i), CTL1 (b, f, j), and CTL2 (c, g, k) individually and together (d, h, l) at 7 (a–d), 18 (e–h), and 39 (i–l) weeks gestation are displayed. (**B**) Higher magnification (scale bar: 20 µm) representative images showing localization of nuclei (a, e, i, m), CTL1 (b, f, j), and CTL2 (c, g, k) individually and together (d, h, l, p) at 7 (a–d), 18 (e–h), and 28 (i–l) weeks of gestation are displayed. A no primary antibody control is shown (m–p) for the 28 week gestation time point. (**C**) Lower (scale bar: 100 µm) and higher (scale bar: 20 µm) magnification representative images showing localization of CTL1 (a,e,i,m), CTL2 (c,g,k,o) individually and together with nuclei (b,f,j,n,d,h,l,p) in term placentas (39 weeks) that did not experienced labor (a–d, i–l) or did experience labor (e–h, m–p) are displayed. STB=syncytial trophoblasts, CTB=cytotrophoblasts, S=stroma, V=fetal vasculature, yellow represents co-localization of CTL1 and CTL2, n=6 for each gestational time point (representative images shown).

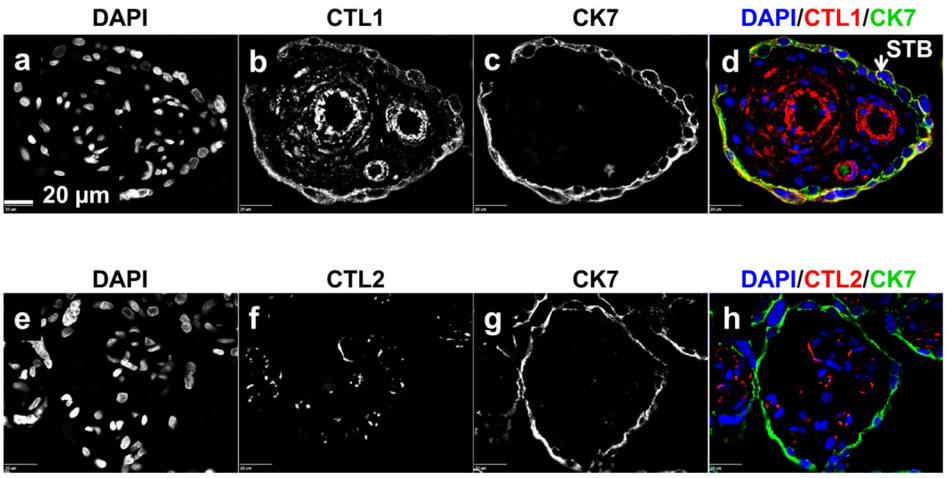


Figure 3. CTL1 localizes to trophoblasts

Immunofluorescence was performed on paraffin embedded tissue sections from the chorionic villi region of human placenta collected at 6–8, 10–12, 18–24, 28–32, 37–40 (labored and unlabored) week gestational time points. Representative images are displayed (shown here: 39 weeks, no labor). Fluorescence from antibodies directed to CTL1 [b, d (red)], CTL2 [f, h (red)], and CK7 [c, d (green), f, h (green)] was imaged with a confocal microscope. STB=syncytial trophoblasts, scale bar: 20 μm, n=6 for each gestational window.

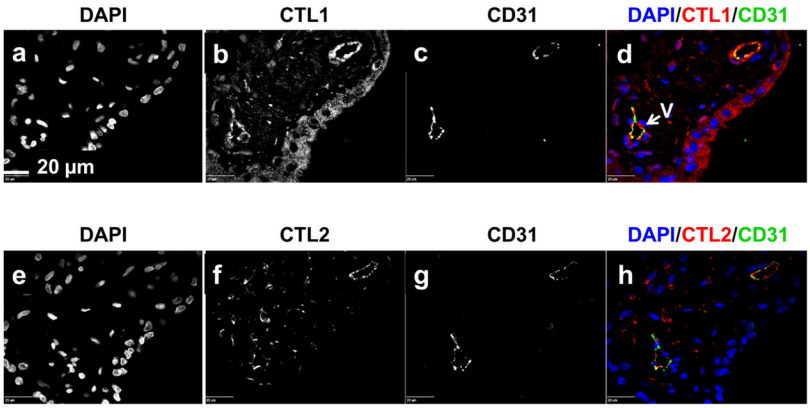


Figure 4. CTL1 and CTL2 localize to fetal endothelial cells

Immunofluorescence was performed on paraffin embedded tissue sections from the chorionic villi region of human placenta collected at 6–8, 10–12, 18–24, 28–32, 37–40 (labored and non-labored) week gestational time points. Representative images are displayed (shown here: 11 weeks). Fluorescence from antibodies directed to CTL1 [b, d (red)], CTL2 [f, h (red)], and CD31 [c, d (green), f, h (green)] was imaged with a confocal microscope. V=fetal vasculature, scale bar: 20 µm, n=6 for each gestational window.